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FORM PCT/RO/101 (Modified) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
0217-0006	
U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	
To Be Assigned	09/744751

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/KR99/00414	30-July-1999	31-July-1998

TITLE OF INVENTION

Lipid Emulsion and Solid Lipid Nanoparticle as a Gene or Drug Carrier

OIPE JAN 29 2001 PATENT & TRADEMARK OFFICE TC1100

APPLICANT(S) FOR DO/EO/US

JEONG, Seo Young; KWON, Ick Chan; and CHUNG, Hesson

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a ☐ are transmitted herewith (required only if not transmitted by the International Bureau)
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

1. Copy of PCT Publication (WO 00/06120);
2. Copy of Notification of Receipt of Demand by Competent International Preliminary Examining Authority;
3. Copy of Amendment Under Article 34 filed with the demand;
4. Copy of Written Opinion; and
5. Postcard.

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
To Be Assigned 09/744751	PCT/KR99/00414	0217-0006

21. The following fees are submitted:.	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :	
☒ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO **$970.00**	
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but Internation Search Report prepared by the EPO or JPO **$840.00**	
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$690.00**	
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$670.00**	
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$96.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$970.00**
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	45 - 20 =	25	x $18.00	**$450.00**
Independent claims	4 - 3 =	1	x $78.00	**$78.00**
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**

TOTAL OF ABOVE CALCULATIONS =	**$1,498.00**
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable).** ☒	**$749.00**
SUBTOTAL =	**$749.00**
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +	**$0.00**
TOTAL NATIONAL FEE =	**$749.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐	**$0.00**
TOTAL FEES ENCLOSED =	**$0.00**
Amount to be: refunded	$
charged	$

☐ A check in the amount of to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

☐ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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D W Woodward
SIGNATURE

David W. Woodward
NAME

35,020
REGISTRATION NUMBER

January 29 2001
DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Jeong, et al.	
	Art Unit: To Be Assigned
Serial No.: To Be Assigned	Examiner: To Be Assigned
Filed: Herewith	Atty. Docket: 0217-0006
For: Lipid Emulsion and Solid Lipid Nanoparticle as a Gene or Drug Carrier	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified application, Applicant herewith respectfully requests the following amendments:

IN THE CLAIMS:

11. The emulsion according to [any of] claim[s] 1, [9 or 10,] further comprising a phospholipid or a non-ionic surfactant.

13. The emulsion according to [any of] claim[s] 1, [9 or 10,] further comprising glycerol or fusogenic peptides.

17. The emulsion according to [any of] claim[s] 1, [9, or 10,] further comprising 1,2-dioleoyl-sn-3-phosphatidylethanolamine, diolein, fatty alcohol, cholesterol or bile salt.

19. The solid lipid nanoparticles according to claim 2 [or 18], further comprising a phospholipid or a non-ionic surfactant.

21. The solid lipid nanoparticles according to claim 2 [or 18], further comprising glycerol or fusogenic peptides.

25. The solid lipid nanoparticles according to claim 2 [or 18], further comprising 1,2-dioleyl-sn-3-phosphatidylethanolamine, diolein, fatty alcohol, cholesterol or bile salt.

52. A complex of the emulsion according to [any of] claim[s] 1, [9 to 17] and a biologically active material selected from the group consisting of DNA, RNA, antisense nucleic acid, ribosome, polynucleotide, and oligonucleotide.

54. The complex according to claim 52 [or 53], further comprising protamine sulfate, histone or cationic polymer.

62. A complex of the solid lipid nanoparticles according to [any of] claim[s] 2, [12 to 19] with a biologically active material selected from the group consisting of DNA, RNA, antisense nucleic acid, ribosome, polynucleotide and oligonucleotide.

64. The complex according to claim[s] 62 [or 63], further comprising protamine sulfate, histone or cationic polymer.

69. The complex according to [any of] claim[s] 62 [to 68], further comprising lipophilic or amphiphilic drug in the fat, wherein the lipophilic or amphiphilic drug is selected from the group consisting of antivirals, steroidal anti-inflammatory drugs, non-steroidal anti-inflammatory drugs, antibiotics, antifungals, vitamins, hormones, retinoic acid, prostaglandins, prostacyclins, anticancer drugs, antimetabolitic drugs, miotics, cholinergics, adrenergic antagonists, anticonvulsants, antianxiety agents, major tranquilizers, antidepressants, anesthetics, analgesics, anabolic steroids, estrogens,

progesterones, glycosaminoglycans, polynucleotides, immunosuppressants and immunostimulants.

REMARKS

It is respectfully requested that the Examiner enter these amendments prior to examining the application on its merits.

Respectfully submitted,

SHANKS & HERBERT

By: DWWoodward

David W. Woodward
Reg. No. 35,020

Date: January 29 2001

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(703) 683-3600

Applicant or Patentee: JEONG, et al.

Appl. or Patent No.: _____ Docket No.: 0217-0006

Filed or Issued: _____

For: Lipid Emulsion and Solid Lipid Nanoparticle as a Gene or Drug Carrier

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 C.F.R. §§ 1.9(f) and 1.27(d)) – NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: KOREA INSTITUTE OF SCIENCE AND TECHNOLOGY
ADDRESS OF ORGANIZATION: 39-1, Hawolgok-dong, Sungbook-ku, Seoul 136-791, Republic of Korea

TYPE OF ORGANIZATION:

[X] UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
[ ] TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. § 501(a) and 501(c)(3))
[ ] NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)
[ ] WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. 501(a) AND 501(c)(3) IF LOCATED IN THE UNITED STATES OF AMERICA
[ ] WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(NAME OF STATUTE _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. § 1.9(e), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention, entitled: Lipid Emulsion and Solid Lipid Nanoparticle as a Gene or Drug Carrier

By inventor(s) Seo Young Jeong, Ick Chan Kwon, and Hesson Chung
described in

[ ] the specification filed herewith
[X] application no. 09/744,751, filed January 29, 2001
[ ] patent no. _____, issued _____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, or by any concern which would not qualify as a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. 1.9(e). *NOTE: Separate verified statements are

required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 C.F.R. § 1.27).

NAME _____
ADDRESS _____

( ) INDIVIDUAL ( ) SMALL BUSINESS CONCERN ( ) NONPROFIT ORGNIZATION

NAME _____
ADDRESS _____

( ) INDIVIDUAL ( ) SMALL BUSINESS CONCERN ( ) NONPROFIT ORGNIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 C.F.R. § 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING PARK, Hokoon

TITLE OF PERSON OTHER THAN OWNER President

ADDRESS OF PERSON SIGNING 39-1, Hawolgok-dong, Sungbook-ku, Seoul 136-791, Republic of Korea

SIGNATURE Park Hokoon DATE Mar. 13, 2001

LIPID EMULSION AND SOLID LIPID NANOPARTICLE AS A GENE OR DRUG CARRIER

DISCLOSURE OF THE INVENTION

The present invention relates to oil-in-water lipid emulsions, a complex between the emulsion and genes, and emulsions loaded with hydrophobic or amphiphilic drugs using biocompatible and biodegradable fats and oils. The present invention also relates to a method of preparing oil-in-water lipid emulsions, a complex between the emulsion and genes, and emulsions loaded with hydrophobic or amphiphilic drugs using biocompatible and biodegradable fats and oils.

The present invention also relates to a method of transferring genes efficiently into cells by using said lipid emulsions.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will become more fully understood from the detailed description given hereinbelow and the accompanying drawings which are given by way of illustration only, and thus are not limitative of the present invention, and wherein:

Figure 1 is a graph showing a correlation between the average droplet size of the lipid emulsion for different oils and the interfacial tension between water and oil;

Figure 2a is a graph showing a correlation between the average droplet size of the lipid emulsion using different triglycerides and the concentration of DLPC;

Figure 2b is a graph showing a correlation between the changes in the average droplet size of the lipid emulsion using tributyrin and the concentration of DLPC;

Figure 2c is a graph showing a correlation between the changes in the average droplet size of the lipid emulsion using tricaproin and the concentration of DLPC;

Figure 2d is a graph showing a correlation between the changes in the average droplet size of the lipid emulsion using tricaprylin and the concentration of DLPC;

Figure 3a is a graph showing a correlation between the average droplet size

of the lipid emulsion using tributyrin and the concentration of the emulsifiers;

Figure 3b is a graph showing a correlation between the average droplet size of the lipid emulsion using tricaproin and the concentration of the emulsifiers;

Figure 3c is a graph showing a correlation between the average droplet size of the lipid emulsion using tricaprylin and the concentration of the emulsifiers;

Figure 4 is an electrophoresis photograph showing a complex formation between DNA and lipid emulsion of the present invention. One microgram of pCMV-beta plasmid and different amounts of lipid emulsion were used to form a complex;

Lane 1: DNA molecular weight marker;

Lane 2: 1 μg of pCMV-beta plasmid [7164 base pairs (bp)];

Lane 3: 2 μl of Lipofectamine and 1 μg of pCMV-beta plasmid;

Lane 4: 4 μg of DOTAP liposome and 1 μg of pCMV-beta plasmid;

Lanes 5, 6, 7 and 8: 1,2,4 and 6 μg, respectively, of DOTAP/squalene lipid emulsion and 1 μg of pCMV-beta plasmid;

Lane 9: 4 μg of DOTAP/soybean oil lipid emulsion and 1 μg of pCMV-beta plasmid;

Lane 10: 4 μg of DOTAP/linseed oil lipid emulsion and 1 μg of pCMV-beta plasmid.

Figure 5 is an electrophoresis photograph of pCMV-beta after the carrier/DNA complexes underwent an exchange reaction with poly-L-aspartic acid;

A) Lipofectamine and DOTAP liposome, B) linseed oil emulsion/pCMV-beta complex, C) soybean oil emulsion/pCMV-beta complex and D) squalene emulsion/pCMV-beta complex;

Lane 1: DNA molecular weight marker;

Lane 2: 1 μg of pCMV-beta plasmid [7164 base pairs (bp)];

Lane 3: carrier/ pCMV-beta complex;

Lanes 4-13: carrier/ pCMV-beta complex incubated with 0.625, 1.25, 2.5, 5.0, 25, 50, 100, 200, 400 and 800 equivalency of poly-L-aspartic acid for an hour. Equivalency in the present invention represents the charge ratio between phosphate group of DNA and carboxylic group of PLAA.

Figure 6 is a transmission electron micrograph of cationic solid lipid nanoparticle, DNA and their complex.

A) pCMV-beta, B) trilaurin solid lipid nanoparticle, C) complex between trilaurin solid lipid nanoparticle and pCMV-beta (1/1 by weight) and D) complex between trilaurin solid lipid nanoparticle and pCMV-beta (2/1 by weight).

B)

Figure 7 is a graph showing the effect of a helper lipid DOPE on the transfection efficiency of liposome and emulsion carriers;

A) DOTAP/DOPE liposome, B) DOTAP/DOPE squalene lipid emulsion

■: no serum, □: 80 % serum.

Figure 8 is a graph showing the effect of a helper lipid diolein on the transfection efficiency of liposome and emulsion carriers;

A) DOTAP/DOPE liposome, B) DOTAP/DOPE squalene lipid emulsion

■ : no serum, □ : 80 % serum.

Figure 9 is a graph showing the changes in the transfection efficiency of liposome and emulsion carriers by adding a non-ionic surfactant, Tween 80;

A) DOTAP/DOPE/Tween 80 liposome, B) DOTAP/DOPE/Tween 80 squalene lipid emulsion

■ : no serum, □ : 80 % serum.

Figure 10 is a graph showing the stability of the lipid emulsion by adding Tween 80;

μ : DOTAP/DOPE squalene lipid emulsion, λ : DOTAP/DOPE/Tween 80 squalene lipid emulsion.

Figure 11 is a graph showing the changes in the transfection efficiency using the lipid carriers by adding protamine sulfate;

A) protamine sulfate B) 4 μg of DOTAP/DOPE/Tween80 liposome and protamine sulfate C) 4 μg of DOTAP/DOPE/Tween80 lipid emulsion and protamine sulfate

λ : no serum, μ : 80 % serum.

Figure 12 is a graph showing the transfection efficiency using different lipid gene carriers with various cell-lines

■ : no serum, □ : 80 % serum.

Figure 13 is a graph showing the difference in *in vitro* release rates of rifampicin from different lipid emulsions;

▽ : PBS, λ :linseed oil emulsion, μ : soybean oil emulsion, τ : squalene emulsion.

Figure 14 is a graph showing the difference in *in vitro* release rates of diclofenac from different lipid emulsions;

▽ : PBS, λ :linseed oil emulsion, μ : soybean oil emulsion, τ : squalene emulsion.

Figure 15 is a graph comparing the *in vivo* transfection efficiencies when 10

μg of DNA was administered intravenously by using different lipid gene carriers.

Figure 16 is a graph comparing the *in vivo* transfection efficiencies when 50 μg of DNA was administered intravenously by using different lipid gene carriers.

Figure 17 is a graph comparing the *in vivo* transfection efficiencies when DNA was administered intravenously by using different lipid gene carriers: effect of emulsifiers with PEG moiety.

Figure 18 is a graph comparing the *in vivo* transfection efficiencies when DNA was administered intravenously by using different lipid gene carriers: effect of protamine sulfate.

Figure 19 is a graph comparing the *in vivo* transfection efficiencies when DNA was administered by intranasal instillation by using different lipid gene carriers.

Figure 20 is a graph showing the difference in the in vitro release rates of diclofenamic acid from different lipid emulsions;

▽ : PBS, λ :linseed oil emulsion, μ : soybean oil emulsion, τ : squalene emulsion.

TECHNICAL FIELD

It is an object of the present invention to provide a method of preparing oil-in-water type lipid emulsion and solid lipid nanoparticles composed of biocompatible materials.

Another object of the present invention is to provide a complex between biologically active material and lipid emulsion comprising non-triglyceride oils and a complex between biologically active material and solid lipid nanoparticle comprising a fat selected from the group comprising triglycerides and ethyl stearate, and the preparation method thereof.

Another object of the present invention is to provide lipid emulsions comprising squalene or squalane loaded with drugs or to provide ethyl stearate solid lipid nanoparticles loaded with drugs and the preparation methods thereof.

The present invention also concerns the method of transferring genes and drugs efficiently into cells by using the lipid emulsions.

BACKGROUND ART

Liposome and emulsion are the two of the well-known non-viral gene carriers

Gene therapy using liposomes are under clinical trials. There are many reports in the past several years regarding liposome-mediated gene delivery.

Triglyceride lipid emulsion, which has been developed recently, may form a complex that maintains physical stability and delivers genes in the presence of serum. These triglyceride emulsions, however, has a low physical stability and loses the ability to transfer genes without a polymeric lipid such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-N[-poly(ethyleneglycol)2000]($PEG_{2000}PE$). The polymeric lipids that may provide physical stability of emulsions include Span, Tween, and Brij series lipids having polyethylene glycol moiety in the lipid headgroup. These polymeric lipids, however, may attenuate the interaction between the lipid emulsion and the gene since the polymeric moiety provides a steric hindrance Therefore, the cationic lipid emulsion containing polymeric lipid could form a complex with DNA when there are 3-4 excess positive charges of emulsions.

To overcome these problems, an object of the present invention is to prepare a stable lipid emulsion without or with a minimal amount of polymeric lipid. Accordingly, we have used different oils that are known to be biocompatible with an acceptable toxicity. Among the lipid emulsions when egg phosphatidylcholine (PC) was used as an emulsifier, we have discovered that the oils that have higher interfacial tension against water forms more stable emulsions than those with a lower interfacial tension. We also have discovered that the correlation between emulsion stability and the oil/water (o/w) interfacial tension has not been systemically studied. There is a small indication that the oils that have smaller o/w interfacial tension may form a more stable emulsion. Therefore, the present inventors tired to provide a scientific background to provide a reason for the correlation between the emulsion stability and the physical properties of oils.

Preliminary experiment to provide a scientific background for the present invention:

Lipid emulsions are generally prepared by dispersing oil in water by use of an emulsifier. There are many theoretical works on how emulsions can be stabilized. While studying the factors to stabilize the lipid emulsions, the present inventors have found that there are only few studies on the effect of the oils that have different physical properties such as lipophilicity and the stability of the resulting emulsions.

To investigate how the lipophilicity of the oils changes the stability of the o/w

emulsions, many different oils were selected to form emulsions by using egg PC as an emulsifier. The lipid emulsions having 10 %(v/v) of oils and 1.2 %(w/v) of egg PC were obtained by 1) preparing a liposome solution by sonicating egg PC in water and 2) adding oil in the liposome solution to sonicate the mixture for 2 min for three times (total 6 min). The size of the particles in the emulsion was measured by photon correlation spectroscopy. The emulsion made with linseed oil has the biggest emulsion droplet sizes whereas the squalene and jojoba bean oil formed emulsions with the smallest droplet sizes. The relationship between the size of the emulsion and the o/w interfacial tension of the oils is shown in Figure 1. The results show that the oil that has bigger o/w interfacial tension forms an emulsion with smaller droplet size. The oils are mainly mixtures of triglycerides and esters of fatty acid/alcohols, except squalene.

In the present invention, emulsions made with squalene or squalane are stable without the use of polymeric lipids. There are some examples in the previous studies on the squalene emulsions. The squalene emulsions are used as vaccine adjuvants (US patent 5,376,369). Squalene was selected as an oil phase in o/w emulsion since it has a low cytotoxicity and is biodegradable. Squalene emulsion was also formulated as a Taxol carrier (US patent 5,407,683). However, this Taxol-loaded squalene emulsion is formulated by forming a self-emulsifying glass by adding surfactant or alcohols and by dispersing it in water. A large amount of ethanol is included in the formulation.

Therefore, the composition and the preparation method are completely different form those of the present invention. In summary, squalene emulsions known up to now are 1) vaccine adjuvants to deliver antigens and 2) Taxol-loaded emulsions having different composition and preparation method from the present invention. Therefore, the lipid emulsion of the present invention to deliver genes and lipophilic or amphiphilic drugs differs from the pre-existing squalene emulsion formulas. Squalene emulsion shows a far superior stability to the emulsions made by castor, soybean, and safflower or sunflower oil. The release rate of lipophilic drug is slower from more stable emulsions. Also, bioavailability of the drug is better for more stable emulsions. Though further study is required to explain how the interfacial tension and the emulsion droplet size is related, the decrease in the interfacial tension by offering different tension gradient by different oils may be a possible explanation.

The present invention is also related to the ethyl stearate solid lipid nanoparticles. Solid lipid nanoparticles made with ethyl stearate, or more broadly the ethyl esters of fatty acid or alcohols with C10-18 straight chains, have never been used as gene carriers. Moreover, the ethyl stearate solid lipid nanoparticles have not been used as a carrier of a lipophilic or amphiphilic drug.

The present invention provides the lipid emulsion made of non-triglycerides including squalene or squalane and 1) cationic emulsifier and 2) phospholipids and lipid with PEG moiety, and the preparation methods thereof. Also, the present invention provides cationic solid lipid nanoparticles to deliver genes or other biologically active materials and the preparation method thereof. The solid lipid nanoparticles of the present invention are suspension of solid fat particles in water. Therefore, it may be freeze-dried to remove water from the system to preserve the fat particles. It has an advantage of preparing drug-loaded solid lipid nanoparticles that can be freeze-dried to increase storage time.

Cationic solid lipid nanoparticle is composed of triglyceride, having C12-18 straight chain hydrophobic tail, which exists as a solid at room and body temperatures and cationic emulsifier. There are no current reports on the positively charged solid lipid nanoparticle as a gene carrier.

Lipid emulsions made of triglycerides have been widely used as a drug delivery system. For instance, there are many different emulsion formulations for cyclosporin, an oligopeptide immunosurppresant. Medium chain triglycerides with C8-12 carbons were used to solubilize cyclosporin in o/w emulsions (US patent 5,660,858). Emulsions, made by alcohol, alkanol, polysorbate 80 and cyclosporin with submicron particle sizes, are reported to be stable and have high bioavailability (WO 97/35,603). According to WO 97/36,610, cyclosporin dissolved in medium chain triglycerides was dispersed in water easily by using propylene carbonate as a co-emulsifier with an average droplet size of *ca.* 100 nm. Although there are many cyclosporin formulations, there are no reports or patents on the emulsion formulation using squalene or squalane, highly pure branched hydrocarbons. Also, there are no reports on the cyclosporin formulation using solid lipid nanoparticles made of ethyl stearate. Squalene, squalane and ethyl stearate are mono-component oil systems Therefore, unlike vegetable oils made of many components, the physical and chemical properties do not vary. Mono-component system provides a better quality control in mass-production Moreover, according to the present invention, squalene

emulsion is more stable having smaller average particle sizes than many other vegetable emulsions. The in vitro drug release experiment also shows that the release of drugs such as rifampicin is sustained with a zero-order release pattern from the squalene emulsion.

The squalene and squalane emulsions and the ethyl stearate solid lipid nanoparticle can be used as a carrier of negatively-charged biologically active materials including DNA when cationic emulsifier was used and as a drug delivery system for lipophilic or amphiphilic drugs. Moreover, the cationic emulsion or solid lipid nanoparticle, loaded with lipophilic anticancer drugs can form a complex with a therapeutic gene in cancer therapy.

MODE(S) FOR CARRYING OUT THE INVENTION

According to the first aspect of the present invention, the present invention provides a lipid emulsion to deliver genes or other biologically active materials comprising a) 2-30 % of oil of non-triglycerides, b) 0.01-20 % of one or more emulsifiers including a cationic surfactant and c) water to 100 %. The lipid emulsion of the present invention can include other additives. The emulsifier in the present invention is required to make the surface charge of the emulsion positive and therefore, it is selected from an emulsifier having a positive charge to form a complex with a negatively charged biologically active material including DNA. A non-ionic surfactant, phospholipid, fatty acid, fatty alcohol, bile salt or cholesterol may be additionally used as an emulsifier.

According to the second aspect of the present invention, the present invention provides a solid lipid nanoparticle to deliver genes or other biologically active materials comprising a) 2-30 % of triglycerides having 10-18 carbons in each hydrophobic tails or ethyl stearate, b) 0.01-20 % of one or more emulsifiers including a cationic surfactant and c) water to 100 %. The lipid emulsion of the present invention can include other additives. The emulsifier in the present invention is required to make the surface charge of the emulsion positive and therefore, it is selected from an emulsifier having a positive charge to form a complex with a negatively charged biologically active material including DNA. A non-ionic surfactant, phospholipid, fatty acid, fatty alcohol, bile salt or cholesterol may be additionally used as an emulsifier.

According to the third aspect of the present invention, the present invention provides a drug-loaded lipid emulsion to deliver a lipophilic or amphiphilic drug comprising a) 2-30 % non-triglycerides oil such as squalene or squalane, b) 0.01-20 % of one or more emulsifiers, c) 0.1-10 % lipophilic or amphiphilic drug and d) water to 100 %. The drug-containing emulsion of the present invention can include other additives. The surface charge of the drug-containing emulsion of the present invention may be positive, zero or negative.

According to the forth aspect of the present invention, the present invention provides a drug-loaded solid lipid nanoparticle to deliver a lipophilic or amphiphilic drug comprising a) 2-30 % of ethyl stearate, b) 0.01-20 % of one or more emulsifiers, c) 0.1-10 % lipophilic or amphiphilic drug and d) water to 100 %. The drug-containing solid lipid nanoparticle of the present invention can include other additives. The surface charge of the drug-containing solid lipid nanoparticle of the present invention may be positive, zero or negative.

The present invention is also related to the method of preparing the lipid emulsion to deliver gene or other biologically active materials to cells. The preparation method of the lipid emulsion according to the present invention include a) a first step of preparing an aqueous phase by mixing 0.01-20 % of one or more emulsifiers including a cationic emulsifier and b) a second step of preparing emulsion by mixing said aqueous phase with 2-30 % of non-triglycerides oil.

The present invention is also related to a method of preparing the solid lipid nanoparticle to deliver gene or other biologically active materials to cells. The preparation method of the solid lipid nanoparticle according to the present invention include a) a first step of preparing an aqueous phase by mixing 0.01-20 % of one or more emulsifiers including a cationic emulsifier and b) a second step of preparing emulsion by mixing said aqueous phase with 2-30 % of fat of triglycerides having 10-18 carbons in each hydrophobic tails or ethyl stearate.

The present invention is also related to a method of preparing the lipid emulsion to deliver lipophilic or amphiphilic drugs to cells. The preparation method of the drug-loaded lipid emulsion according to the present invention include a) a first step of preparing an aqueous phase by mixing 0.01-20 % of one or more emulsifiers, b) a second step of preparing oil phase by mixing 0.1-10 % of lipophilic or amphiphilic drug and 2-30 % of non-triglycerides oil and b) a third step of preparing drug-loaded emulsion by mixing the aqueous and oil phases prepared in the first and

second steps, respectively.

The present invention is also related to a method of preparing the solid lipid nanoparticle to deliver lipophilic or amphiphilic drugs to cells. The preparation method of the drug-loaded solid lipid nanoparticle according to the present invention include a) a first step of preparing an aqueous phase by mixing 0.01-20 % of one or more emulsifiers, b) a second step of mixing 0.1-10 % of lipophilic or amphiphilic drug and 2-30 % of fat such as ethyl stearate and b) a third step of preparing drug-loaded solid lipid nanoparticle by mixing the aqueous and oil phases prepared in first and second steps, respectively.

It is also possible to prepare a drug-loaded lipid emulsion or solid lipid nanoparticle by first preparing an oil or fat phase, respectively, comprising oil or fat, respectively, emulsifier and drugs and subsequently by mixing the oil phase with water.

Also, the present invention is related to the preparation method of the lipid emulsion to deliver biologically active materials including genes to cells. The preparation method includes a) a first step of preparing an oil phase by mixing 2-30 % of one or more emulsifier including a cationic emulsifier with 0.1-20 % of non-triglycerides oil and b) a second step of mixing the oil phase with water.

The present invention is also related to the preparation method of the solid lipid nanoparticle to deliver biologically active materials including genes to cells. The preparation method includes a) a first step of preparing a fat phase by mixing 0.1-20 % of one or more emulsifier including a cationic emulsifier with 2-30 % fat of triglycerides having 10-18 carbons in each hydrophobic tails and ethyl stearate and b) a second step of mixing the fat phase with water.

Also the present invention is related to a method of preparing the drug-loaded lipid emulsion. The preparation method includes a) a first step of preparing an oil phase by mixing 0.1-20 % of one or more emulsifier and 0.1-10 % of lipophilic or amphiphilic drug with 2-30 % of non-triglycerides oil and b) a second step of mixing the oil phase with water.

The present invention is also related to a method of preparing the drug-loaded solid lipid nanoparticle. The preparation method includes a) a first step of preparing an fat phase by mixing 0.1-20 % of one or more emulsifier and 0.1-10 % of lipophilic or amphiphilic drug with 2-30 % fat such as ethyl stearate and b) a second step of mixing the oil phase with water.

The lipid emulsion according to the present invention represents a heterogeneous mixture of two or more immiscible liquid stabilized by the use of a surfactant or an emulsifier.

The solid lipid nanoparticle according to the present invention represents a solid-state fat dispersed in a liquid stabilized by the use of a surfactant or an emulsifier.

The non-triglycerides of the present invention include squalene and squalane.

The fat in the solid lipid nanoparticle of the present invention includes ethyl ester of alcohol or acid having a straight chain having 10-18 carbons, and preferably ethyl stearate.

The emulsifier according to the present invention may additionally include phospholipid or non-ionic surfactant. The cationic surfactant used as emulsifiers of the present invention include 1,2-dimyristoyl-3-trimethylammonium-propane (DMTAP), 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP), 1,2-distearoyl-3-trimethylammonium-propane (DSTAP), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dimyristoyl-3-dimethylammonium-propane (DMDAP), 1,2-dipalmitoyl-3-dimethylammonium-propane (DPDAP), 1,2-dilauroyl-3-dimethylammonium-propane (DLDAP), 1,2-distearoyl-3-dimethylammonium-propane (DSDAP), dimethyldioctadecylammonium chloride (DDAB), N-[1-(1,2-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and 1,2-dioleoyl-3-ethylphosphocholine (DOEPC) and other cationic phospholipid. The cationic emulsifiers are required to deliver genes since they may not only interact with negatively charged DNA to form a complex but also enhance the interaction with cells with an excess positive charge.

The phospholipids according to the present invention include phosphatidylcholine (PC) and derivatives thereof, phosphatidylethanolamine (PE) and derivatives thereof and phosphatidylserine (PS). Among the phospholipids, L-α-dioleoyl phosphatidylethanolamie (DOPE) is well known to disturb the endosomal membrane allowing DNA to enter cytoplasm and as a consequence to increase the transfection efficiency.

A fusogenic lipid including DOPE or diolein may be additionally used as an emulsifier. Also, fatty acid, fatty alcohol, cholesterol or bile salt may be additionally used as an emulsifier to increase the physical stability of the lipid emulsion or solid lipid nanoparticle.

Non-ionic surfactants in the present invention include poloxamers (also known as a pluronic: a copolymer of polyoxyethylene and polyoxypropylene), sorbitan esters (Span), polyoxyethylene-sorbitan fatty acid esters (Tween) and polyoxyethylene ethers (Brij).

Additionally, the present invention may comprise hydrophilic polymers, polymeric lipid where the hydrophilic polymers are covalently bonded to a phospholipid or polymerizable lipid such as diacetylated phospholipid. Examples of the hydrophilic polymers that may be used in the present invention are polyoxyethylene, polyethyloxazoline and polyethyleneglycol (PEG). The polymeric lipids enhance the steric stability of the emulsion, and also PEG with a small molecular weight is a well-known fusogenic agent.

The compositions of the present invention may also comprise an osmotic pressure regulator such as glycerol.

The present invention may also comprise low-molecular weight polyethylene glycol (average molecular weight in the range of 500-1000) and fusogenic peptide, such as HA gp 41, to improve the transfection efficiency.

The present invention may also comprise materials such as glycolipid, lipopeptide, antibody, and ligand for receptors, viral protein to target specific cells or organs.

The present invention may also comprise polycations such as protamine sulfate, histone and polylysine to condense DNA.

The present invention may comprise mono- or poly-anions to alter the interaction between lipid carrier and DNA.

The biologically active material which can be used in the present invention include DNA, ribonucleic acid (RNA), antisense nucleic acid, ribosome, polynucleotide, oligonuclotide, or other pharmaceutical drugs.

In the case the biologically active material to be deliver is a nucleic acid such as DNA, a carrier must have a positive charge to form a complex with the nucleic acid and also must be within the appropriate size range.

The positively charged lipid emulsion or solid lipid nanoparticle of the present invention may be loaded with lipophilic or amphiphilic drugs.

The lipid emulsion or solid lipid nanoparticle of the present invention may additionally include a hydrophilic drug in the aqueous phase.

For an *in vivo* application, the serum effect must also be considered. The

lipid emulsion of the present invention is physically stable and has high transfection efficiency in the presence of serum.

The lipid emulsion that can deliver biologically active materials such as DNA according to the present invention is prepared by mixing a) 2-30 % of non-triglycerides oil and b) an aqueous phase comprising 0.01-20 % of one or more emulsifiers including a cationic surfactant and additionally 0.01-10% of hydrophilic polymer or phospholipid having a hydrophilic polymeric moiety.

The solid lipid nanoparticle that can deliver biologically active materials such as DNA according to the present invention is prepared by mixing a) 2-30 % of triglycerides having 10 -18 carbons in the hydrophobic tail or ethyl stearate which is heated above its melting temperature and b) a heated aqueous phase comprising 0.01-20 % of one or more emulsifiers including a cationic surfactant and additionally 0.01-10% of hydrophilic polymer or phospholipid having a hydrophilic polymeric moiety. Upon mixing, the temperature of the aqueous and oil phases must be maintained above the melting temperature of the oil phase.

The present invention also provides a method of preparing drug-loaded lipid emulsion for the *in vivo* delivery of lipophilic or amphiphilic drugs.

The drug loaded lipid emulsion of the present invention is prepared by mixing a) an oil phase comprising 2-30 % of non-triglycerides oil and 0.1-10 % of lipophilic or amphiphilic drug and b) an aqueous phase comprising 0.01-20 % of one or more emulsifiers and additionally 0.01-10% of hydrophilic polymer or phospholipid having a hydrophilic polymeric moiety.

The present invention also provides a method of preparing drug-loaded solid lipid nanoparticle for the *in vivo* delivery of lipophilic or amphiphilic drugs.

The drug loaded solid lipid nanoparticle of the present invention prepared by mixing a) an oil phase comprising 2-30 % of triglycerides oil having 10-18 carbons in the hydrophobic tail or ethyl stearate which is heated above its melting temperature and 0.1-10 % of lipophilic or amphiphilic drug and b) an aqueous phase comprising 0.01-20 % of one or more emulsifiers and additionally 0.01-10% of hydrophilic polymer or phospholipid having a hydrophilic polymeric moiety. Upon mixing, the temperature of the aqueous and oil phases must be maintained above the melting temperature of the oil phase.

It is also possible to prepare the drug-loaded lipid emulsion or solid lipid nanoparticle by solubilizing emulsifiers and lipophilic or amphiphilic drug in the oil

phase completely and subsequently mixing the oil and aqueous phases.

The lipid emulsion that can deliver biologically active materials such as DNA according to the present invention is prepared by mixing a) an oil phase composed of 2-30 % of non-triglycerides oil and 0.01-20 % of one or more emulsifiers including a cationic surfactant and b) an aqueous phase comprising 0.01-10% of hydrophilic polymer or phospholipid having a hydrophilic polymeric moiety.

The solid lipid nanoparticle that can deliver biologically active materials such as DNA according to the present invention is prepared by mixing a) an oil phase comprising 2-30 % of fat of triglycerides having 10-18 carbons in the hydrophobic tail or ethyl stearate which is heated above its melting temperature, and 0.01-20 % of one or more emulsifiers including a cationic surfactant and b) a heated aqueous phase comprising 0.01-10% of hydrophilic polymer or phospholipid having a hydrophilic polymeric moiety. Upon mixing, the temperature of the aqueous and oil phases must be maintained above the melting temperature of the oil phase.

The present invention also provides a method of preparing drug-loaded lipid emulsion for the *in vivo* delivery of lipophilic or amphiphilic drugs.

The drug loaded lipid emulsion of the present invention is prepared by mixing a) an oil phase comprising 2-30 % of non-triglycerides oil, 0.01-20 % of one or more emulsifiers and 0.1-10 % of lipophilic or amphiphilic drug and b) an aqueous phase comprising 0.01-10% of hydrophilic polymer or phospholipid having a hydrophilic polymeric moiety.

The present invention also provides a method of preparing drug-loaded solid lipid nanoparticle for the in vivo delivery of lipophilic or amphiphilic drugs.

The drug loaded solid lipid nanoparticle of the present invention is prepared by mixing a) an oil phase comprising 2-30 % of triglycerides oil having 10-18 carbons in the hydrophobic tail or ethyl stearate which is heated above its melting temperature, 0.01-20 % of one or more emulsifiers and 0.1-10 % of lipophilic or amphiphilic drug and b) an aqueous phase comprising 0.01-10% of hydrophilic polymer or phospholipid having a hydrophilic polymeric moiety. Upon mixing, the temperature of the aqueous and oil phases must be maintained above the melting temperature of the oil phase.

The aqueous phase is prepared by preparing liposome according to the method well known in the field. The aqueous and oil phases are mixed by using the method well known in the field. The prepared aqueous and oil phases are heated to

solubilize the components and mixed by using tools such as a homogenizer, sonicator or microfluidizer.

The present invention also is related to a method of transferring biologically active materials such as DNA into cells by using lipid emulsions made of non-triglyceride and solid lipid nanoparticles made of saturated triglycerides or ethyl stearate.

The present invention also relates to a method of in vivo delivery of lipophilic or amphiphilic drugs by using lipid emulsions made of non-triglyceride and solid lipid nanoparticles made of saturated triglycerides or ethyl stearate.

The lipid emulsion and solid lipid nanoparticle according to the present invention deliver the biologically active materials into cells wherein the cell is selected from the group consisting of white blood cells, fibroblasts, cancer cells, cells infected with virus, epithelial cells, endothelial cells, muscle cells, liver cells, endocrine cells, neural cells, dermal cells, germ cells, oocytes, sperms, hematopoietic cells, fetal cells, M cells, Langerhans islet cells, macrophages, plant cells, animal cells, and immortalized cell lines.

The method of delivering the biologically materials into the target cells according to the present invention is to form a complex between the lipid emulsion or solid lipid nanoparticle with biologically active material.

When the lipid emulsion of the present invention is used as the carrier of genetic materials, it can be administered intravenously, intramuscularly, intranasally, intratracheally, subcutaneously, parenterally, by a topical administration, or direct administration to a specific organ.

The lipophilic and/or amphiphilic drug that may be loaded in the lipid emulsion or the solid lipid nanoparticle includes antivirals, steroidal anti-inflammatory drugs (SAID), non-steroidal anti-inflammatory drugs (NSAID), antibiotics, antifungals, vitamins, hormones, retinoic acid, prostaglandins, prostacyclins, anticancer drugs, antimetabolitic drugs, miotics, cholinergics, adrenergic antagonists, anticonvulsants, antianxiety agents, major tranquilizers, antidepressants, anesthetics, analgesics, anabolic steroids, estrogens, progesterones, glycosaminoglycans, polynucleotides, immunosuppressants and immunostimulants.

The invention will be further illustrated by the following examples, but is not limited to the examples given.

Example 1 Preparation of lipid emulsion using egg phosphatidylcholine as an emulsifier

After mixing egg phosphatidylcholine (eggPC) with water at a concentration of 12 mg/ml and incubating for at least 10 minutes for hydration, the mixture was sonicated using a probe type sonicator (High intensity ultrasonic processor, 600-Watt) for 2 minutes to prepare a liposome solution. Lipid emulsions were prepared with 10 %(v/v) different oils as listed in and the liposome solution by sonication for 2 minutes for 3 times (6 minutes total). The size of the emulsion particles were measured by using Malvern Zetasizer(Malvern Instruments Limited, England) after diluting the emulsion by 300 times in deionized distilled water. The size of the emulsiion particles one day and 20 days after preparation is presented in Table 1 to show the size change of the emulsion droplets with time. The values represent an average from 3 measurements with a single sample. As can be seen in the Table, squalene, a non-triglyceride lipid forms more stable emulsion with a smaller average droplet size than other emulsions listed. The emulsions so prepared were stored at 4 °C until further experiments.

Table 1

Oil	Size(nm) one day after preparation	Size (nm) 20 days after preparation
Castor oil	246.3	290.2
Coconut oil	246.3	278.2
Corn oil	261.0	298.7
Cottonseed oil	263.0	402.8
Borage oil	247.1	418.0
Fish oil	247.0	271.3
Jojoba bean oil	224.0	236.8
Lard oil	282.7	307.9
Linseed oil	354.9	-*
Olive oil	263.0	306.1
Peanut oil	256.8	341.3
Safflower seed oil	283.6	275.9
Sesame oil	263.3	327.8
Soybean oil	249.7	272.0
Squalene	191.7	216.2

* The particle size increased beyond measurable values.

Example 2. Preparation of cationic lipid emulsions using oils having different o/w interfacial tensions

Lipid emulsions were prepared using various oils (10 %(v/v)) and DOTAP as an emulsifier DOTAP was mixed with water at a concentration of 24 mg/ml to solubilize the lipids at 37 °C. The lipid solution was sonicated by using a probe type sonicator for 2 minutes to form a liposome solution. Various oils (10 %(v/v)) were added to the liposome solutions and sonicated for 2 minutes 3 times (total 6 minutes) to form lipid emulsions. The size and the zeta potential of the lipid emulsions were measured by using Malvern Zetasizer (n=3). The results are listed in Table 2. The lipid emulsion made of oils having higher o/w interfacial tension has a smaller average droplet sizes than those made of oils having lower o/w interfacial tension. The emulsion particle sizes were below 200 nm with an exception of linseed oil emulsion The zeta-potential of the emulsions were 48.4 ± 8.5 mV (n=3) The emulsions were kept at 4 °C until further experiments.

Table 2

	Oil	Size (nm)	Zeta-potential (mV)
Liposome		108.5 ± 45.1	48.4 ± 8.5
Emulsion A	Linseed oil	220.4 ± 23.1	50.1 ± 5.4
Emulsion B	Soybean oil	204.1 ± 18.4	57.7 ± 6.7
Emulsion C	Squalane	168.4 ± 10.4	
Emulsion D	Squalene	157.5 ± 8.9	64.5 ± 7.2

Example 3. Stability of lipid emulsions made of various oils in the presence of serum

The stability of the DOTAP liposomes and emulsions were compared by measuring the emulsion particle sizes in the presence of serum. The result of the measurements was listed in Table 3. Even with 0.5 % of serum, the sizes of the liposomes increased by 2.7 times. In the case of lipid emulsions, there are no apparent changes in the sizes in the presence of serum. One of the problems in delivering genes using liposomes was the formation of large insoluble aggregates that can attenuate the transfection efficiency. The lipid emulsions of the present invention overcame such a problem by maintaining the small size with narrow size distribution in the presence of serum.

Table 3. Stability of lipid gene carriers in the presence of serum

	Oil	Size (nm)	
		no serum	0.5 % serum
Liposome		108.5 ± 45.1	295.3 ± 85.9
Emulsion A	Linseed oil	220.4 ± 23.1	261.5 ± 12.4
Emulsion B	Soybean oil	204.1 ± 18.4	201.3 ± 11.1
Emulsion D	Squalene	157.5 ± 8.9	174.0 ± 5.5

Example 4 Preparation of cationic solid lipid nanoparticles

Lipid emulsions were prepared using various oils (10 %(v/v)) and DOTAP as an emulsifier. DOTAP was mixed with water at a concentration of 24 mg/ml to solubilize the lipids at 37 °C. The lipid solution was sonicated by using a probe type

sonicator for 2 minutes to form a liposome solution. Trilaurin or ethyl stearate according to Table 1 were heated to 50 °C. Melted oils (10 %(v/v)) were added to the liposome solutions and sonicated for 2 minutes for 3 times (total 6 minutes) to form lipid emulsions at 50 °C. The lipid emulsion was slowly cooled at room temperature to solidify melted oils and as a resultant to form solid lipid nanoparticles. The sizes and the zeta potential of the lipid emulsions were measured by using Malvern Zetasizer (n=3). The results are listed in Table 4. The emulsions were kept at 4 °C until further experiments.

Table 4

Fat	Size(nm)
Trilaurin	181.2 ± 13.2
Ethyl stearate	183.2 ± 1.4

Example 5. Cell culture and isolation of plasmid DNA

Cell Culture

COS-1 cells (kidney, SV40 transformed, African green monkey) were grown in 5 % CO_2 incubator in DMEM supplemented with 10 % fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin.

Isolation of plasmid DNA

A plasmid pCMV-CAT encoding a chloramphenicol acetyl transferase driven by a human cytomegalovirus immediate-early promoter was purchased from Invitrogen (Groningen, Netherlands). The pCMV-beta encoding a β-galactosidase driven by a human cytomegalovirus immediate-early promoter was purchased from Clontech, Inc.

The plasmids were amplified in a *E. coli* DH5-α strain and purified by using a Quiagen mega-kit (Quiagen Inc., Chatsworth, CA) according to the manufacturer's instruction. DNA purity was determined by agarose gel electrophoresis and by measuring optical density. DNA having O.D. $_{260}$/O.D $_{280}$ ≥1.8 were used in this study

Example 6: Complex formation between cationic lipid emulsion and DNA

The complex formation between DNA and the lipid emulsion prepared in

Example 2 was observed by using a gel electrophoresis technique. Lipofectamine and DOTAP liposomes were used as controls. DNA (1 μg) was mixed with appropriate amounts of lipid carriers in a test tube, and the complexes were incubated for 30 minutes at room temperature. The complexes were loaded on a 1 % agarous gel containing ethidium bromide and electrophoresed in Tris-acetate-EDTA buffer solution at pH 8.0. The gel was visualized under a UV light as shown in Figure 4.

As shown in Figure 4, 2 μl of Lipofectamine formed a complex with 1 μg of DNA. Likewise the emulsions also formed emulsion/DNA complexes effectively.

Example 7: Protection of DNA against PLAA

DNA complexes were formed with the Lipofectamine, DOTAP liposomes and cationic lipid emulsions as in example 6. Different concentrations of poly-L-aspartic acid (PLAA) were added to the complex. Since PLAA is an anionic polymer, it can dissociate the anionic DNA from the complex. Therefore, the dissociation of DNA from the complex in the presence of PLAA is an indicator of the strength of the complex. DNA was dissociated from the Lipofectamine/DNA and DOTAP liposome/DNA complexes at or above the equivalency between PLAA/DNA of 1.25. On the other hand, the BRC 001-DNA complex remained stable in at the equivalence of 640 The result indicates that the lipid emulsion/DNA complex is stronger than liposome/DNA complexes in the presence of an anionic competitor. Therefore, it is expected that the emulsion carrier can exhibit superior transfection efficiency in the presence of biological barriers including serum or mucus layer.

Example 8: Gene transfection efficiency in COS-1 cell line by cationic lipid emulsions

COS-1 cells were seeded at 1 x 10^4 cells in a 96-well plate *ca.* 12 hours prior to transfection. pCMV-beta plasmid DNA (0.5 μg) were mixed with DOTAP liposome and lipid emulsions as example 2 at amounts to contain 2 μg of DOTAP The mixtures were diluted in 2 μl of serum free DMEM medium. The mixtures were completely shaken 10 times by inversion during 30 min incubation period. After cleaning the COS-1 cells with serum free RPMI 1640, 160 μl serum free media and carrier-DNA mixture were added to cells. After a 1 h incubation at 37 °C in a 5 % carbon dioxide incubator, the cells were washed with serum free DMEM to remove

the remaining carrier-DNA complex in the solution. The cells were incubated in DMEM containing 10% serum for 24 hours. The transfected cells were harvested after the incubation. Cells were washed with 200 μl PBS, harvested with 50 μl of lysis solution (0.1% Triton X-100, 250 mM Tris, pH8.0), and lysed by means of a freeze-thaw cycle. In each well, 50 μl of PBS contained 0.5 % of bovine serum albumin. The substrate solution (150 μl of 1mg/ml chloramphenicol red galactopyranoside) was added at room temperature for an hour for enzymatic reaction. A recombinant β-galactosidase was used as a standard. The optical density was measured at 570 nm (n=3). The transfection efficiency is expressed as the relative β-galactosidase activity as shown in Table 5.

Table 5. Transfection efficiency and cytotoxicity using lipid emulsions having different oils.

	oil	Transfection efficiency (mU/well)		Cell proliferation rate (%)
		no serum	80 % serum	
Liposome		0.73 ± 0.13	0.04 ± 0.01	78.4 ± 13.5
Emulsion A	Linseed oil	0.22 ± 0.10	0.08 ± 0.02	77.1 ± 12.4
Emulsion B	Soybean oil	0.21 ± 0.07	0.12 ± 0.02	85.7 ± 11.7
Emulsion C	Squalane	0.30 ± 0.08	0.18 ± 0.08	85.9 ± 12.2
Emulsion DI	Squalene	0.37 ± 0.09	0.22 ± 0.07	89.5 ± 10.2

Example 9 Effect of serum on transfection efficiency in COS-1 cell line

To test the serum effect on DNA transfection efficiency, carrier-DNA complexes were prepared in DMEM without serum. The cells were washed with DMEM without serum. Transfection was performed in the presence of 80 % serum (in 160 μl of DMEM) according to the method in Example 8. The transfection efficiency in the presence of 80 % serum is listed in Table 5.

Example 10. Cytotoxicity

Live cells is quantified by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide (MTT) assay. A tetrazolium ring opening reaction can take place only in the presence of the live cells with active mitochondria. This

yellow-to-purple reaction is quantified by using the scanning multiwell spectrophotometer (ELISA reader).

The COS1 cells were harvested from exponential phase culture by trypsinization, counted and plated in 96-well microplates. A concentration of 1 x 10^4 cells/well was found suitable. Plates were incubated at 37°C for 1 day under a humidified atmosphere. The lipid emulsions were diluted in growth medium to a final volume of 200 μl and added to the cells and incubated for 24 hours. After exposure with a lipid emulsion, the cells were washed twice with PBS. MTT was dissolved in PBS, filter-sterilized and stored at 4°C until further experiment. Two hundred (200) μl of fresh media and 50 μl of 0.5 % MTT solution were added to each well and incubated for 4 hours at 37°C to allow intracellular metabolization. Formazan crystals were dissolved by adding 200 μl of dimethyl sulfoxide (DMSO). Sorensen's glycine buffer (25 μl) was added. The absorbance was measured at 570 nm on an Elisa reader [SOFTmax PRO (Molecular Devices corporation, California, U S.A.)] as shown in Table 5.

The transfection efficiency is lower for the lipid emulsions than for the liposomes without serum. However, the transfection efficiency of the liposome decreased by 18.2 times upon adding serum. In contrast, the transfection efficiency of the lipid emulsion decreased on by 1.6-2.7 times upon adding serum. Among the lipid emulsions, the transfection efficiency of the squalene emulsion was better than other emulsion carriers with or without serum. The squalene emulsion has a superior physical stability with or without serum. This physical stability may provide an ability to deliver genes to cells in the presence of many destabilizing factors as *in vivo* applications. Moreover, the cytotoxicity of the emulsions was lower than liposomes. The squalene emulsion had a lowest cytotoxicity having more than 89 % of cell proliferation rate.

Example 11 Complex formation between cationic solid lipid nanoparticle and DNA

The cationic solid lipid nanoparticle made of trilaurin in Example 4 and DNA were mixed at different ratios in a buffer solution and negatively stained with 1 % uranyl acetate to observe the complex under transmission electron microscope at a magnification ratio of 50,000. Figure 6A is pure DNA. Figures 6C and 6D represents the solid lipid nanoparticle/DNA complexes.

Example 12. Transfection with cationic solid lipid nanoparticle

Cationic solid lipid nanoparticle prepared with trilaurin was used for the in vitro transfection. The β-galactosidase activity was 0.24 ± 0.07 and 0.11 ± 0.02 mU/well without or with 80 % serum, respectively. The cell proliferation rate was 82.6 ± 15.6 % indicating low cytotoxicity of the solid lipid nanoparticles. The results indicate that the solid lipid nanoparticles can be used as a gene carrier.

Example 13 Increase in the transfection efficiency by adding DOPE as a helper lipid to the cationic lipid emulsion.

Squalene emulsions were prepared by using an additional emulsifier, DOPE to increase transfection efficiency. The helper lipid, DOPE has a fusogenicity at room temperature and is known to disturb endosomal membrane to increase the transfection efficiency.

The liposome and squalene emulsion were prepared as in Example 2, except the emulsifier was 24 mg/ml DOTAP/DOPE mixtures instead of pure DOTAP. The weight ratio between DOTAP and DOPE were 11:1, 7:1, 5:1, 3:1, 5:3, 1:1, 1:3 and 1:5. Transfection was performed by following the method in Example 8. Also, the serum effect was studied using the method in Example 9. The results are illustrated in Figure 7. The transfection efficiency was maximum at DOTAP/DOPE ratio of 5:1 for liposome and lipid emulsion. At a higher DOPE concentration, the transfection efficiency decreases probably due to the instability of the lipid carriers and the complexes resulted by the addition of the DOPE.

Example 14 Increase in the transfection efficiency by adding diolein as a helper lipid to the cationic lipid emulsion.

Squalene emulsions were prepared by using an additional emulsifier, diolein, to increase transfection efficiency. The present inventors have tested the possibility of using diolein, which has not been used as a helper lipid, to increase the transfection activity.

The liposome and squalene emulsion were prepared as in Example 2, except the emulsifier was DOTAP/diolein mixture (9:1 by weight) instead of pure DOTAP. Transfection was performed by following the method in Example 8. Also, the serum effect was studied using the method in Example 9. The results are illustrated in Table 6.

The transfection efficiency was increased by adding diolein for liposome and lipid emulsion. Interestingly, cytotoxicity of the liposome decreased upon adding diolein. This result is in contrast with the increase in cytotoxicity of the liposome upon adding DOPE. The decrease in the cytotoxicity may come from the fact that diolein activates protein kinase C which regulates cell function. Therefore, unlike DOPE, diolein may be a better helper lipid that increases the transfection efficiency with a lowered cytotoxicity.

Table 6. Influence of diolein on Transfection efficiency and cell proliferation rate.

	Oil	Emulsifier	Size (nm)	Transfection efficiency (mU/well)		Cell proliferation rate (%)
				no serum	80% serum	
Liposome	-	DOTAP	148.5 ± 44.1	0.73 ± 0.13	0.04 ± 0.01	78.4 ± 13.5
	-	DOTAP/ DIOLEIN	185.2 ± 21.1	0.98 ± 0.12	0.07 ± 0.04	83.4 ± 14.8
Lipid emulsion	Squalene	DOTAP	145.1 ± 18.4	0.35 ± 0.09	0.20 ± 0.04	82.7 ± 10.4
	Squalene	DOTAP/ DIOLEIN	165.2 ± 19.2	0.46 ± 0.07	0.22 ± 0.07	85.6 ± 11.1

Example 15 Increase in the transfection efficiency by adding DOPE and diolein simultaneously as helper lipids to the cationic lipid emulsion.

Both DOPE and diolein in Examples 13 and 14, respectively, were used as helper lipids to DOTAP in squalene emulsions in transfection. The weight ratio between DOTAP and DOPE was set at 5:1 and the amount of diolein was changed. The squalene emulsion was prepared as in Example 2, except the emulsifiers were DOTAP, DOPE and diolein mixtures instead of pure DOTAP. The weight ratio between (DOTAP+DOPE) and diolein were 1:0, 7:1, 5:1, 3:1, 1:1 and 1:3. Transfection was performed by following the method in Example 8. Also, the serum effect was studied using the method in Example 9. The results are illustrated in Figure 9.

The transfection efficiency was maximum at (DOTAP+DOPE)/DOPE ratio of 5:1. By adding DOPE and diolein, DOPE and diolein may have provided fusogenicity and diolein may have acted as a protein kinase C activator.

Transfection activity, however, decreased at higher concentrations indicating that diolein changes the stability of emulsion/DNA complex.

Example 16 Transfection efficiency and cytotoxicity by adding an emulsifier with polyethylene glycol moiety.

Emulsifier with polyethylene glycol (PEG) moiety may increase the stability of emulsions by providing a steric hindrance. Also, low molecular weight PEG unit may also provide a fusogenicity. Therefore, different PEG-lipids were used as a co-emulsifier to form emulsions. Squalene emulsions were prepared by using 24 mg/ml DOTAP/DOPE (5:1 by weight) and different polymeric emulsifiers. Different amounts (10, 20, 30 and 50 %(w/w) of the weight of DOTAP/DOPE mixture) of polymeric emulsifiers, Tween 80, $PEG_{2000}PE$, HCO 60 and Pluronic F68, were added to from emulsions. Transfection and cytotoxicity studies were performed by following the methods in Examples 8 and 10. Depending on the type of polymeric lipid, optimum transfection efficiency was obtained at different DOTAP concentrations. The results in Table 7 show the optimum efficiency for each emulsion.

In the case of liposomes, the transfection efficiency in the presence of 80 % serum decreased significantly by adding polymeric lipids. In contrast, the transfection efficiency with 80 % serum was at least 50 % of that without serum when lipid emulsions were used as gene carriers. The transfection efficiency increased for liposome and emulsion upon adding Tween 80. However, other polymeric lipids lowered the transfection efficiency due to the steric hindrance of the polymeric moiety As a result, larger amounts of the carrier were needed for the interaction between cationic lipid and DNA with an increased cytotoxicity. Therefore, Tween 80 was the lipid of choice that provides emulsion stability without hindering the interaction between emulsion and DNA.

Table 7.

Formulation	Non-ionic surfactant	DOTAP (mg)*	Size (nm)	Transfection efficiency (mU/well)		Cell proliferation rate (%)
				No serum	80% serum	
Liposome A	-	2	154.4 ± 25.1	0.90 ± 0.31	0.08 ± 0.01	78.9 ± 18.5
Liposome B	Tween80	3	145.6 ± 12.1	1.51 ± 0.28	0.12 ± 0.05	76.0 ± 11.3
Liposome C	$PEG_{2000}PE$	5	151.41 ±19.4	0.54 ± 0.12	0.04 ± 0.02	82.0 ± 12.3
Liposome D	HCO60	5	140.2 ± 0.98	0.50 ± 0.10	0.06 ± 0.02	85.0 ± 10.7
Emulsion A	-	2	200.1 ± 08.7	0.62 ± 0.15	0.38 ± 0.11	89.3 ± 15.4
Emulsion B	Tween80	4	186.3 ± 13.2	1.05 ± 0.18	0.52 ± 0.13	79.8 ± 18.2
Emulsion C	$PEG_{2000}PE$	8	190.2 ± 10.4	0.41 ± 0.10	0.23 ± 0.09	81.8 ± 14.5
Emulsion D	HCO60	8	162.0 ± 08.9	0.33 ± 0.11	0.24 ± 0.07	83.2 ± 12.3
Emulsion E	F68	10	232.2 ± 12.3	0.20 ± 0.09	0.10 ± 0.06	78.3 ± 15.7

* Amount of DOTAP required to form a complex with 0.5 g DNA that shows maximum transfection efficiency.

Example 17. Increase in the transfection efficiency by adding Tween 80

Squalene emulsions were prepared by using 24 mg/ml DOTAP/DOPE (5:1 by weight) and different polymeric Tween 80. The amount of Tween 80 was 0, 5, 10, 15 and 20 %(w/w) in addition to DOTAP/DOPE. Transfection and was performed by following the method in Example 8. The results are shown in Figure 9. The transfection efficiency increased as the amount of Tween 80 increased up to 10 %. At higher than 10 % of Tween 80, however, the efficiency decreased probably due to an increase of the steric hindrance of the polyethylene glycol moiety.

Example 18 Increase in the emulsion stability by adding Tween 80

To investigate whether Tween 80 increases the emulsion stability, squalene emulsions having 24 mg/ml DOTAP/DOPE (5:1 by weight) with or without 10 %(w/w) Tween 80 as in Example 17 were prepared. Emulsions were diluted by 300 fold in phosphate buffered saline with 0.5 % serum and 0.1 % sodium azide to measure absorbance at 600 nm (n=3). The absorbance immediately after dilution was set to 100 % as shown in Figure 10. Unlike the emulsion made without Tween 80, the

absorbance of the emulsion with Tween 80 did not change with time indicating that the emulsion stability increased by adding Tween 80.

Example 19 Preparation methods

Lipid emulsion having 10 %(w/w) Tween 80 of Example 17 was prepared by two different preparation methods; microfluidization and sonication. Size and transfection efficiency were compared. Preparation method of the sonicated emulsion was same as Example 2. The method using microfluidization was as follows. Oil and aqueous phases were heated to 70 °C to solubilize the components completely before mixing the two phases. The emulsion was prepared by mixing the two solutions with a high-speed homogenizer (T-25-Ultra-Turrax, S25-18G, IKA Werke, Janke & Kunkel GmbH & Co KG, Germany) at 8000 rpm for 10 min The mixture was passed 10 times through a Microfluidizer (Microfluidics Co., Newton, MA) with an exit air pressure of 80 psi. The emulsion so prepared was stored at 4 °C until further use. The size of the emulsion particles was measured by using Malvern Zetasizer after diluting the emulsion by 300 times in water as in Example 1. Transfection was performed as in Example 8. The results are listed in Table 8.

Table 8. Characteristics of lipid emulsions made by different preparation methods.

Method of preparation	Size (nm)	Transfection efficiency (mU/well)	
		No serum	80% serum
Microfluidization	178.5 ± 12.7	1.10 ± 0.20	0.54 ± 0.14
Sonication	186.3 ± 13.2	1.05 ± 0.18	0.52 ± 0.13

Emulsions made by two different methods produced similar results (n=3) It is desirable to use microfluidization method for mass-production and to use sonication for small-scale preparation.

Example 20 Effect of pre-treatment with protamine sulfate on the size of the complex

Protamine sulfate is a polycationic protein and forms a protamine sulfate/DNA complex by interacting with and condensing anionic DNA. Also protamine sulfate has a nucleus targetting moiety that can promote a transfer of the complex to

nucleus to increase the transfection activity.

In forming a complex between emulsion and DNA, protamine sulfate was mixed with DNA before adding an emulsion solution.

Protamine sulfate, at amounts of 0.5, 1.0 and 1.5 μg/well, were added to DNA 15 min before adding the emulsion. Half an amount of the emulsion used in Example 6 (2 μg of DOTAP in the emulsion) was added to the protamine sulfate/DNA mixture. The size of the emulsion particles was measured by using Malvern Zetasizer after diluting the emulsion by 300 times in water as in Example 1. Table 9 shows that the size of the complex does not increase by adding protamine sulfate. Addition of protamine sulfate may lower the amount of cationic lipid emulsion and as a result may lower the emulsion related cytotoxicity.

Table 9.

Formulation	emulsifier	DNA	Protamine sulfate	Size (nm)
Liposome	DOTAP/DOPE	-	-	108.5 ± 15.1
	DOTAP/DOPE	+	-	174.2 ± 08.9
	DOTAP/DOPE	**+**	**+**	**100.2 ± 06.9**
	DOTAP/DOPE/Tween80	-	-	120.4 ± 13.1
	DOTAP/DOPE/Tween80	+	-	138.4 ± 10.1
	DOTAP/DOPE/Tween80	**+**	**+**	**112.2 ± 04.9**
Lipid emulsion	DOTAP/DOPE	-	-	121.1 ± 05.1
	DOTAP/DOPE	+	-	179.4 ± 05.9
	DOTAP/DOPE	**+**	**+**	**119.2 ± 03.9**
	DOTAP/DOPE/Tween80	-	-	130.9 ± 04.9
	DOTAP/DOPE/Tween80	+	-	163.5 ± 05.5
	DOTAP/DOPE/Tween80	**+**	**+**	**125.4 ± 03.9**

Example 21. Increase in transfection efficiency by adding protamine sulfate.

Emulsion/DNA complex was made after pre-treatment with protamine sulfate as in Example 20, and transfection was performed as in Example 8. Transfection efficiency in the presence of serum was also determined by following the method in Example 9. The results are shown in Figure 11. Transfection efficiency by using emulsion or protamine sulfate alone was 7-10 times lower than that by using emulsion/DNA complex pre-treated with DNA. The highest transfection efficiency was obtained with 1.0 μg protamine sulfate.

Interestingly, pre-treatment with protamine sulfate may not increase the transfection efficiency when liposome was used instead of emulsion. Moreover, transfection efficiency was maintained in the presence of serum when lipid emulsion was used as a gene carrier.

Example 22 Transfection efficiency in different cell lines

Lipid emulsion containing DOTAP/DOPE/Tween80 as in Example 19 was used as a transfection reagent in different cell lines using the method in Example 8 except that CV-1 or NIH3T3 cell lines was used instead of COS-1. Figure 12 shows that the lipid emulsion may be used as gene carriers for these cell lines.

Example 23 Preparation of lipid emulsion loaded with rifampicin

Oil phase comprising 1g of oils in Table 10, 100 mg egg PC and 80 mg $PEG_{2000}PE$ were heated to 55 °C to solubilize the components completely Rifampicin (10 mg) was added to the oil phase. The emulsion was prepared by adding 10 ml PBS and by sonicating for 2 min. The size of the emulsion particles was measured by using Malvern Zetasizer after diluting the emulsion by 300 times in water as in Example 1 (Table 10)

Table 10.

Oil	Size (nm)
Linseed oil	226.5±3.12
Soybean oil	218.1±2.83
Squalene	224.0±3.28

Example 24. In vitro release of rifampicin from lipid emulsions

Three milliliters of emulsions prepared in Example 23 were put in dialysis bags, each end tightened by closures. The emulsion-filled bags were sunk in 10 ml PBS contained in 50 ml conical tubes. The conical tubes were put in shaking water bath at 37 °C. As a comparative example, release rate of rifampicin in PBS was compared. Concentration of rifampicin was measured by fluorescence spectroscopy.

As shown in Figure 13, the release rate was slowest from squalene emulsion by showing 0^{th} order release pattern. Release rate was fastest for the rifampicin solution followed by linseed oil emulsion and by soybean oil emulsion.

Example 25 Preparation of lipid emulsion loaded with diclofenac sodium

Emulsions were prepared as in Example 23 except that 3 mg diclofenac sodium was loaded instead of rifampicin. The size of the emulsion particles was measured by using Malvern Zetasizer after diluting the emulsion by 300 times in water as in Example 1

Table 11.

Oil	Size (nm)
Linseed oil	210 0±0.61
Soybean oil	222.5±2.39
Squalene	235.8±0.36

Example 26 In vitro release of diclofenac sodium from lipid emulsions

In vitro release of diclofenac sodium from the emulsions prepared in Example 25 was performed by following the method in Example 26. As a comparative example, release rate of diclofenac sodium in PBS was compared. Concentration of diclofenac sodium was measured by HPLC. Release of diclofenac was faster than that of rifampicin since diclofenac was more hydrophilic.

As shown in Figure 14, the release rate was slowest from squalene emulsion than other emulsions. However, the difference in the release rate is much less pronounced than in case of rifampicin release. Also, release of diclofenac solution was the fastest of all.

Example 27 Estimation of lethal dose

Squalene emulsion prepared in Exmaple 2 was used to estimate the lethal dose. 200 micoliters of emulsions diluted by 1/2, 1/4, 1/8 and 1/16 were injected intravenously to Balb/C mice (groups of 6 mice) to observe the survival rate after 24 hours. The results are shown in Table 12. The dose where half of the mice in the group die (LD_{50}) is approximately 1.6 g DOTAP/kg.

Table 12.

Dilution ratio	DOTAP (mg)	Squalene (mg)	Percent survival
1/2	2.4	8	402
1/4	1.2	4	16.6
1/8	0.6	2	66.6
1/16	0.3	1	100

Example 28 Systemic gene delivery by intraveous administration of emulsion/DNA complex (*In vivo* experiment)

Emulsion/DNA complexes as well as liposome/DNA complexes were delivered systemically by intravenous administration. Complexes between 1.7 μl lipid emulsions prepared in Example 2 and10 μg pCMV-luc + were injected intravenously through the tail vein of 30 g Balb/C mouse. For comparison, liposome/DNA complex and naked DNA were also administered. The mice were sacrificed 22 hours after the injection to analyze the expression of luciferase in each organ. Heart, liver, lung, kidney and spleen were obtained for analysis. Expression of luciferase was measured by following the protocols provided by Promega. Briefly, each organ was homogenized after adding 4 μl of lysis buffer per 1 mg tissue. The ground tissue underwent two freeze-thaw cycles and centrifuged for 2 min at 10,000 g to obtain clear supernatent solution. The supernatant solution was stored at –20 °C until analysis. Ten microliters of the solution was mixed with 100 μl of buffer solution for luciferase analysis to measure relative light unit. As shown in Figure 15, the expression rate in each organ, especially in the lung, was pronounced with squalene emulsion as a gene carrier.

Example 29 Systemic gene delivery by intraveous administration of emulsion/DNA complex (*In vivo* experiment)

In vivo experiments were performed as in Example 28 except that complexes having 10.5 μl lipid emulsions and 50 μg DNA was used. For comparison, liposome/DNA complexes and naked DNA were also administered. The mice were sacrificed 22 hours after the injection to analyze the expression of luciferase in each organ. Heart, liver, lung, kidney and spleen were obtained for analysis. As shown in Figure 16, the expression rate in each organ, especially in the lung, was pronounced with squalene emulsion as a gene carrier.

Example 30 Systemic gene delivery by intraveous administration of emulsion/DNA : Effect of emulsifiers with PEG moiety

In vivo experiments were performed by following the method in Example 28. DNA (10 μg) was complexed with lipid emulsions prepared as in Example 18. Amount of emulsions in the complexes was controlled to show maximum transfection efficiency *in vitro* in Example 18. For comparison, lipsomes having same lipid composition was complexed with DNA for the intraveous injection. The mice were sacrificed 22 hours after the injection to analyze the expression of luciferase in each organ. Heart, liver, lung, kidney and spleen were obtained for analysis. When DOTAP/DOPE and DOTAP/DOPE/Tween 80 were used as emulsifier systems, emulsions showed higher transfection efficiency than the liposomes used The DOTAP/DOPE/Tween 80 emulsion, had a high in vivo transfeciton efficiency especially in the lung.

Example 31. Systemic gene delivery by intraveous administration of emulsion/DNA : Effect of emulsifiers with PEG moiety

In vivo experiments were performed by following the method in Example 28. DNA (pCMV-luc+, 10 μg) was complexed with 30 μg of protamine sulfate and incubated for 15 min at room temperature. Appropriate amount of lipid emulsion prepared as in Example 18 was added by following the method in Example 21 to form a complex. Amount of emulsions in the complex was controlled to show maximum transfection efficiency *in vitro* in Example 18. For comparison, lipsomes having same lipid composition was complexed with DNA for the intraveous injection. The mice were sacrificed 22 hours after the injection to analyze the expression of

luciferase in each organ. Heart, liver, lung, kidney and spleen were obtained for analysis. When DOTAP/DOPE and DOTAP/DOPE/Tween 80 were used as emulsifier systems, emulsions showed higher transfection efficiency than the liposomes used. The DOTAP/DOPE/Tween 80 emulsion, had a high in vivo transfeciton efficiency especially in the lung.

Example 32. Gene delivery by nasal instillation of emulsion/DNA

Emulsion/DNA complexes in Examples 2, 13 and 16 were administered by nasal administration. Complex between DNA (pCMV-luc+, 20 μg) and emulsion was administered to Balb/c Mice (30 g). For comparison, lipsome/DNA complex as well as naked DNA were administered. The mice were sacrificed 20 hours after the injection to analyze the expression of luciferase in the nasal cavity and lung. Expression of luciferase was measured by following the protocols provided by Promega. As shown in Figure 19, squalene emulsions showed prominently higher luciferase expression rate than liposome or naked DNA. Interestingly, squalene emulsion made with DOTAP/DOPE had higher luciferase expression in nasal cavity whereas DOTAP/DOPE/Tween 80 was more efficient in the lung.

Example 33 Preparation of lipid emulsion loaded with diclofenamic acid

Emulsions were prepared as in Example 23 except that 3 mg diclofenamic acid was loaded instead of rifampicin. The size of the emulsion particles was measured by using Malvern Zetasizer after diluting the emulsion by 300 times in water as in Example 1.

Table 13

Oil	Size (nm)
Linseed oil	213.2 ± 3.03
Soybean oil	240.1 ± 1.14
Squalene	239.0 ± 1.23

Example 34 In vitro release of diclofenac sodium from lipid emulsions

In vitro release of diclofenac sodium from the emulsions prepared in Example 32 was performed by following the method in Example 24. As a comparative example, release rate of diclofenac sodium in PBS was compared. Concentration

of diclofenac sodium was measured by HPLC.

Diclofenamic acid are more hydrophobic than its salt form. As shown in Figure 20, the release rate was slowest from squalene emulsion than other emulsions.

Example 35. Preparation of solid lipid nanoparticles loaded with cyclosporin

Oil phase comprising 1g of ethyl stearate, 100 mg egg PC and 80 mg $PEG_{2000}PE$ was heated to 55 °C to solubilize the components completely. Cyclosporin (20 mg) was added to the oil phase at 55 °C. The solid lipid nanoparticles were prepared by adding 10 ml PBS and by sonicating for 2 min. The size of the emulsion particles was measured by using Malvern Zetasizer after diluting the emulsion by 300 times in water as in Example 1 (Table 10). Also, emulsions were prepared as in Example 23 except that 20 mg cyclosporin was loaded instead of rifampicin.

Table 14.

Formulation	Size (nm)
Linseed oil emulsion	199.6 ± 2.6
Soybean oil emulsion	200.5 ± 0.7
Ethyl stearate SLN	180.1 ± 0.7

Example 36 Freeze-drying of solid lipid nanoparticles loaded with cyclosporin

The solid lipid nanoparticles loaded with cyclosporin were freeze dried for convenient storage. Solid lipid nanoparticles resuspended in water had an average particle size of *ca.* 500 nm.

Claims;

1. An oil-in-water lipid emulsion for delivering biologically active material selected from the group consisting of DNA, RNA, antisense nucleic acid, ribosome, polynucleotide and oligonucleotide, comprising: 2-30% of non-triglyceride oil; 0.01-20% of one or more cationic lipid transfection agent; and, water to 100%.

2. Solid-lipid nanoparticles for delivering biologically active material selected from the group consisting of DNA, RNA, antisense nucleic acid, ribosome, polynuleotide and oligonucleotide, comprising: 2-30% of fat of triglycerides having 10-18 carbons in each hydrophobic tail or ethyl stearate; 0.01-20% of one or more cationic lipid transfection agent; and, water to 100%.

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5. A method of preparing an oil-in-water lipid emulsion for delivering biologically active material selected from the group consisting of DNA, RNA, antisense nucleic acid, ribosome, polynucleotide and oligonucleotide, comprising: a) a first step of preparing an aqueous phase by mixing 0.01-20% of one or more cationic lipid transfection agent with water and b) a second step of preparing emulsion of said aqueous phase with 2-30% of non-triglyceride oil.

6. A method of preparing solid lipid nanoparticles for delivering biologically active material selected from the group consisting of DNA, RNA, antisense nucleic

acid, ribosome, polynucleotide and oligonucleotide, comprising: a) a first step of preparing an aqueous phase by mixing 0.01-20% of one or more cationic lipid transfection agent with water and b) a second step of mixing said aqueous phase with 2-30% of fat of triglycerides having 10-18 carbons in each hydrophobic tail or ethyl stearate.

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9. The emulsion according to claim 1, further comprising 0.01-10% of a hydrophilic polymer or hydrophilic polymeric lipid.

10. The emulsion accordign to claim 1, wherein the non-triglycerides is squalene or squalane.

11. The emulsion according to any of claims 1, 9 or 10, further comprising a phospholipid or a non-ionic surfactant.

12. The emulsion according to claim 1, wherein the cationic lipid transfection agent is selected from the group consisting of:

1,2-dimyristoyl-3-trimethylammonium-propane,

1,2-dipalmitoyl-3-trimethylammonium-propane,

1,2-distearoyl-3-trimethylammonium-propane,

1,2-dioleoyl-3-trimethylammonium-propane,

1,2-dimyristoyl-3-dimethylammonium-propane,

1,2-dipalmitoyl-3-dimethylammonium-propane,

1,2-dilauroyl-3-dimethylammonium-propane,

1,2-distearoyl-3-dimethylammonium-propane,

1,2-dipalmitoyl-3-trimethylammonium-propane,

N-[1-(1,2-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride,

1,2-dioleoyl-3-ethylphosphocholine, and other cationic lipids.

13. The emulsion according to any of claims 1, 9, or 10, further comprising glycerol or fusogenic peptides.

14. The emulsion according to claim 13, wherein the fusogenic peptide is polyethylene glycol of MW.500-1000 or HA gp 41.

15. The emulsion according to claim 9, wherein the hydrophilic polymer is selected from the group consisting of polyoxyethylene, polyethyloxazoline and polyethyleneglycol.

16. The emulsion according to claim 11, wherein the phospholipid is selected from the group consisting of phosphatidylcholin, phosphatidylethanolamine, phosphatidylserine, diacetylenic phospholipid and derivative thereof and the non-ionic surface active agent is selected from the group consisting of poloxamer, sorbitan ester, polyoxyethylene-sorbitan fat acid ester and polyoxyethylene ethers.

17. The emulsion according to any of claims, 1, 9, or 10, further comprising 1,2-dioleoyl-sn-3-phosphatidylethanolamine, diolein, fatty alcohol, cholesterol

or bile salt.

18. The solid lipid nanoparticles according to claim 2, further comprising 0.01-10% of a hydrophilic polymer or hydrophilic polymeric lipid.

19. The solid lipid nanoparticles according to claim 2 or 18, further comprising a phospholipid or a non-ionic surfactant.

20. The solid lipid nanoparticle according to claim 2, wherein the cationic lipid transfection agent is is selected from the group consisting of:

1,2-dimyristoyl-3-trimethylammonium-propane,

1,2-dipalmitoyl-3-trimethylammonium-propane,

1,2-distearoyl-3-trimethylammonium-propane,

1,2-dioleoyl-3-trimethylammonium-propane,

1,2-dimyristoyl-3-dimethylammonium-propane,

1,2-dipalmitoyl-3-dimethylammonium-propane,

1,2-dilauroyl-3-dimethylammonium-propane,

1,2-distearoyl-3-dimethylammonium-propane,

1,2-dipalmitoyl-3-trimethylammonium-propane,

N-[1-(1,2-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride,

1,2-dioleoyl-3-ethylphosphocholine, and other cationic lipids.

21. The solid lipid nanoparticles according to claim 2 or 18, further

comprising glycerol or fusogenic peptides.

22. The solid lipid nanoparticles according to claim 21, wherein the fusogenic peptide is polyethylene glycol of MW.500-1000 or HA gp 41.

23. The solid lipid nanoparticles according to claim 18, wherein the hydrophilic polymer is selected from the group consisting of polyoxyethylene, polyethyloxazoline and polyethyleneglycol.

24. The solid lipid nanoparticles accordign to claim 19, wherein the phospholipid is selected from the group consisting of phosphatidylcholin, phosphatidylethanolamine, phosphatidylserine, diacetylenic phospholipid and derivative thereof and the non-ionic surface active agent is selected from the group consisting of poloxamer, sorbitan ester, polyoxyethylene-sorbitan fat acid ester and polyoxyethylene ethers.

25. The solid lipid nanoparticles according to claim 2 or 18, further comprising 1,2-dioleoyl-sn-3-phosphatidylethanolamine, diolein, fatty alcohol, cholesterol or bild salt.

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52. A complex of the emulsion according to any of claims 1, 9 to 17 and a biologically active material selected from the group consisting of DNA, RNA, antisense nucleic acid, ribosome, polynuleotide, oligonucleotide.

53. The complex according to claim 52, further comprising glycolipid, lipopeptide, antibody, ligand for receptors or viral protein to target specific cells or organs.

54. The complex according to claim 52 or 53, further comprising protamine sulfate, histone or cationic polymer.

55. The complex according to claim 54, wherein cationic polymer is polylysine.

56. The complex according to claim 52, further comprising monovalent or multivalent salt.

57 The complex according to claim 53, wherein the cell is selected from the group consisting of white blood cells, fibroblasts, cancer cells, cells infected with virus, epithelial cells, endothelial cells, muscle cells, liver cells, endocrine cells, neural cells, dermal cells, germ cells, oocytes, sperms, hematopoietic cells, fetal cells, M cells, Langerhans islet cells, marcophages, plant cells, animal cells, and immortalized cell lines.

58. The complex according to claim 52, wherein the complex is to be transferred to cells via intravenous, intramuscular, intratracheal, intranasal,

subcutaneous, parenteral or topical administration or via direct administration to a specific organ.

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60. The complex according to claim 52, further comprising lipophilic or amphiphilic drug in an oil phase, wherein the lipophilic or amphiphlic drug is selected from the group consisting of antivirals, steroidal anti-inflammatory drugs, non-steroidal anti-inflammatory drugs, antibiotics, antifungals, vitamins, hormones, retinoic acid, prostaglandins, prostacyclins, anticancer drugs, antimetabolitic drugs, miotics, cholinergics, adrenergic antagonists, anticonvulsants, antianxiety agents, major tranquilizers, antidepressants, anesthetics, analgesics, anabolic steroids, estrogens, progesterones, glycosaminoglycans, polynucleotides, immunosuppressants and immunostimulants.

61. The complex according to claim 60 wherein the anticancer drug is taxol, paclitaxel or flourourcil.

62. A complex of the solid lipid nanoparticles according to any of claims 2, 12 to 19 with a biologically active material selected from the group consisting of DNA, RNA, antisense nucleic acid, ribosome, polynucleotide and oligonucleotide.

63. The complex according to claim 62, further comprising glycolipid, lipopeptide, antibody, ligand for receptors or viral protein to target specific cells or organs.

64. The complex according to claims 62 or 63, further comprising protamine

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sulfate, histone or cationic polymer.

65. The complex according to claim 64, wherein the cationic polymer is polylysine.

66. The complex according to claim 62, further comprising monovalent or multivalent salt.

67. The complex according to claim 63, wherein the cell is selected from the group consisting of white blood cells, fibroblasts, cancer cells, cells infected with virus, epithelial cells, endothelial cells, muscle cells, liver cells, endocrine cells, neural cells, dermal cells, germ cells, oocytes, sperms, hematopoietic cells, fetal cells, M cells, Langerhans islet cells, marcophages, plant cells, animal cells, and immortalized cell lines.

68. The complex according to claim 62, wherein the complex is to be transferred to cells via intravenous, intramuscular, intratracheal, intranasal, subcutaneous, parenteral or topical administration or via direct administration to a specific organ.

69. The complex according to any of claims 62 to 68, further comprising lipophilic or amphiphilic drug in the fat, wherein the lipophilic or amphiphlic drug is selected from the group consisting of antivirals, steroidal anti-inflammatory drugs, non-steroidal anti-inflammatory drugs, antibiotics, antifungals, vitamins, hormones, retinoic acid, prostaglandins, prostacyclins, anticancer drugs, antimetabolitic drugs, miotics, cholinergics, adrenergic antagonists, anticonvulsants, antianxiety agents, major tranquilizers, antidepressants, anesthetics, analgesics, anabolic steroids, estrogens, progesterones,

glycosaminoglycans, polynucleotides, immunosuppressants and immunostimulants.

70. The complex according to claim 69, wherein the anticancer drug is taxol, paclitaxel or fluorouracil.

71. The method according to claim 5, wherein the aqueous phase further comprises 0.01-10% of a hydrophilic polymer or hydrophilic polymeric lipid.

72. The method according to claim 6, wherein the aqueous phase further comprises 0.01-10% of a hydrophilic polymer or hydrophilic polymeric lipid.

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75. The method according to claim 5, wherein the cationic lipid transfection agent is added in the oil phase instead of in an aqueous phase.

76. The method according to claim 6, wherein the cationic lipid transfection agent is added in melted fat instead of in an aqueous phase.

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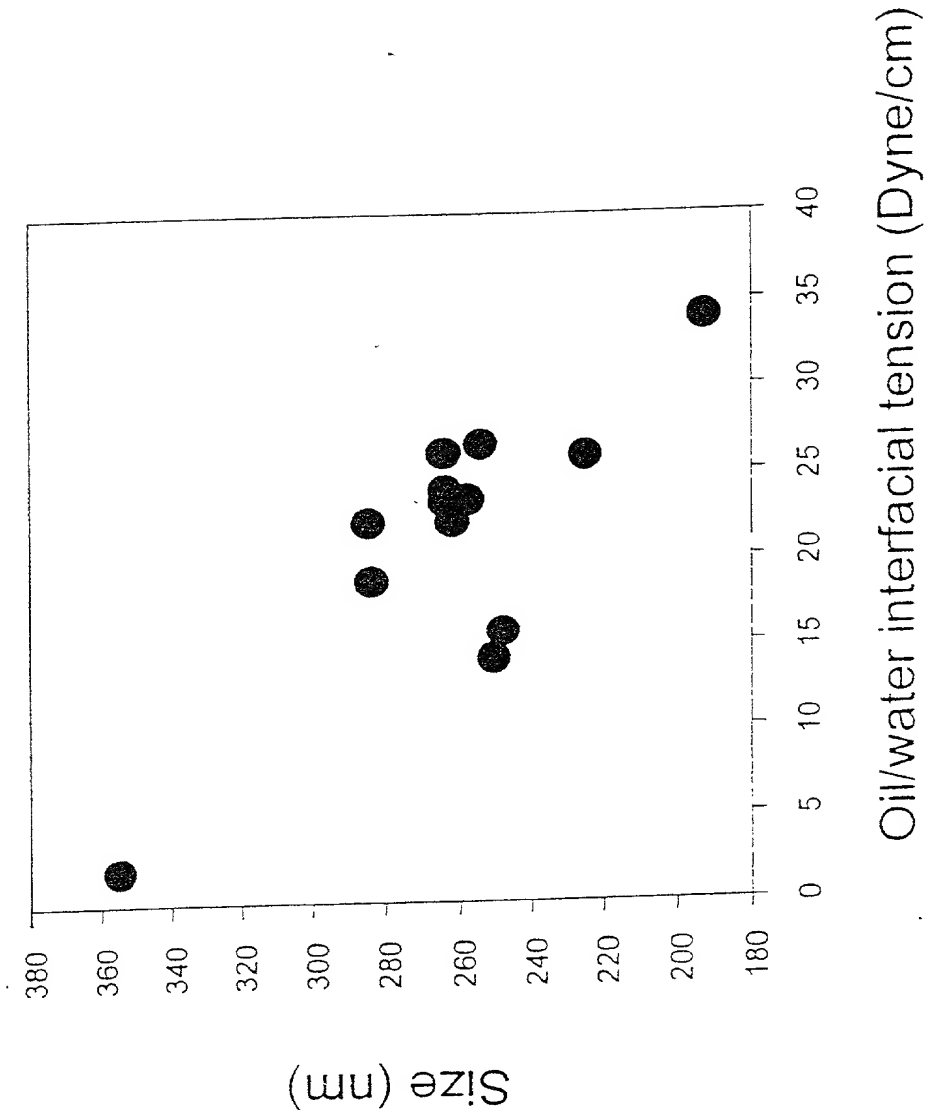
79. The complex according to claim 60, wherein the immunosuppressant is cyclosporin A.

80. The complex according to claim 69, wherein the immunosuppressant is cyclosporin A.

FIG.1


Size (nm)
380
360
340
320
300
280
260
240
220
200
180
0
5
10
15
20
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30
35
40
Oil/water interfacial tension (Dyne/cm)

FIG.2A

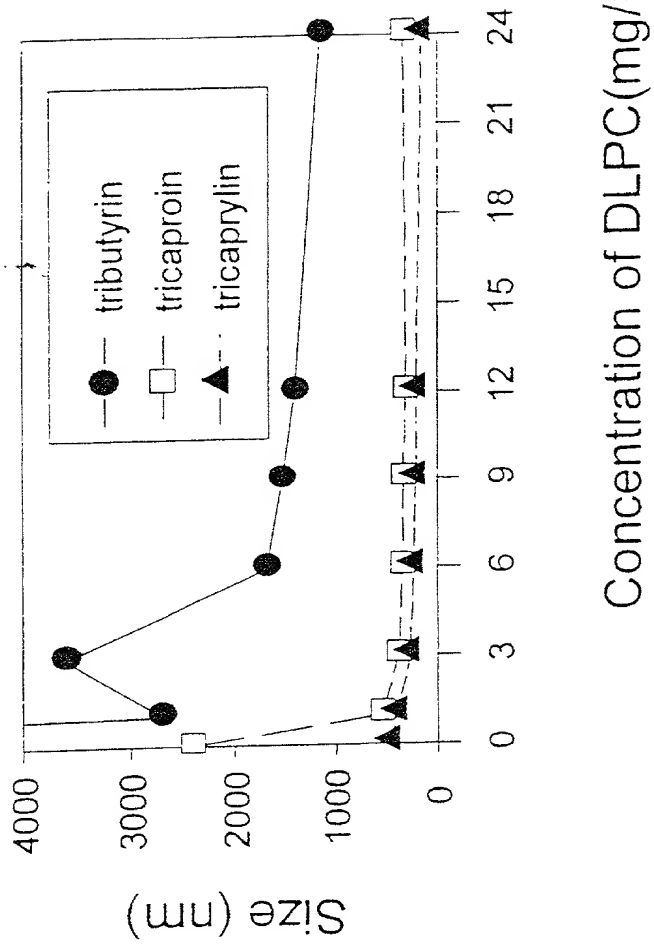

tributyrin
tricaproin
tricaprylin
Size (nm)
4000
3000
2000
1000
0
0
3
6
9
12
15
18
21
24
Concentration of DLPC(mg/ml)

FIG.2B

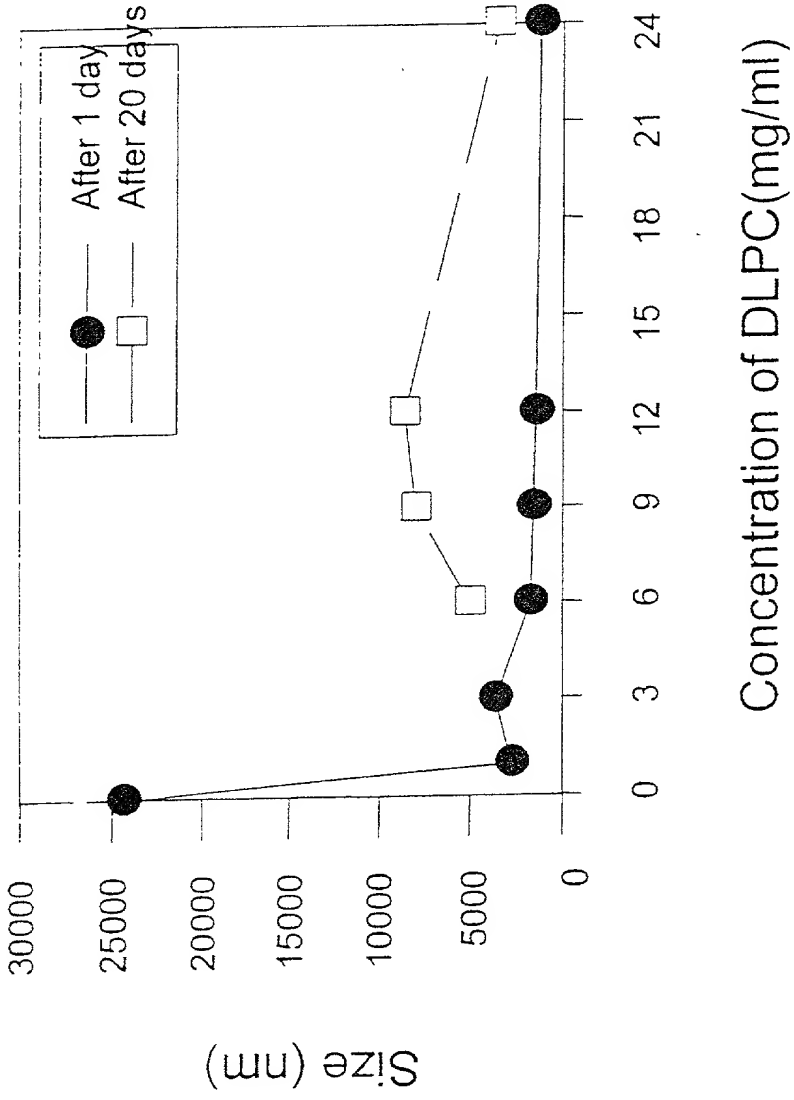

After 1 day
After 20 days
30000
25000
20000
15000
10000
5000
0
Size (nm)
0
3
6
9
12
15
18
21
24
Concentration of DLPC(mg/ml)

FIG.2C

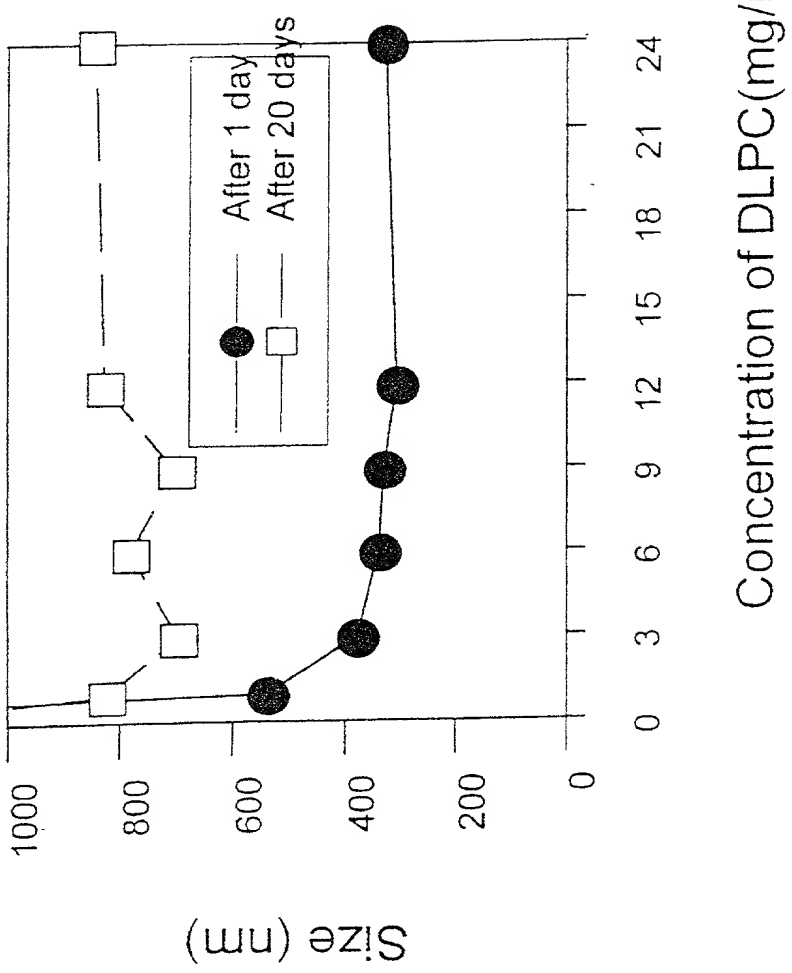

Size (nm)
1000
800
600
400
200
0
0
3
6
9
12
15
18
21
24
Concentration of DLPC(mg/ml)
After 1 day
After 20 days

FIG.2D

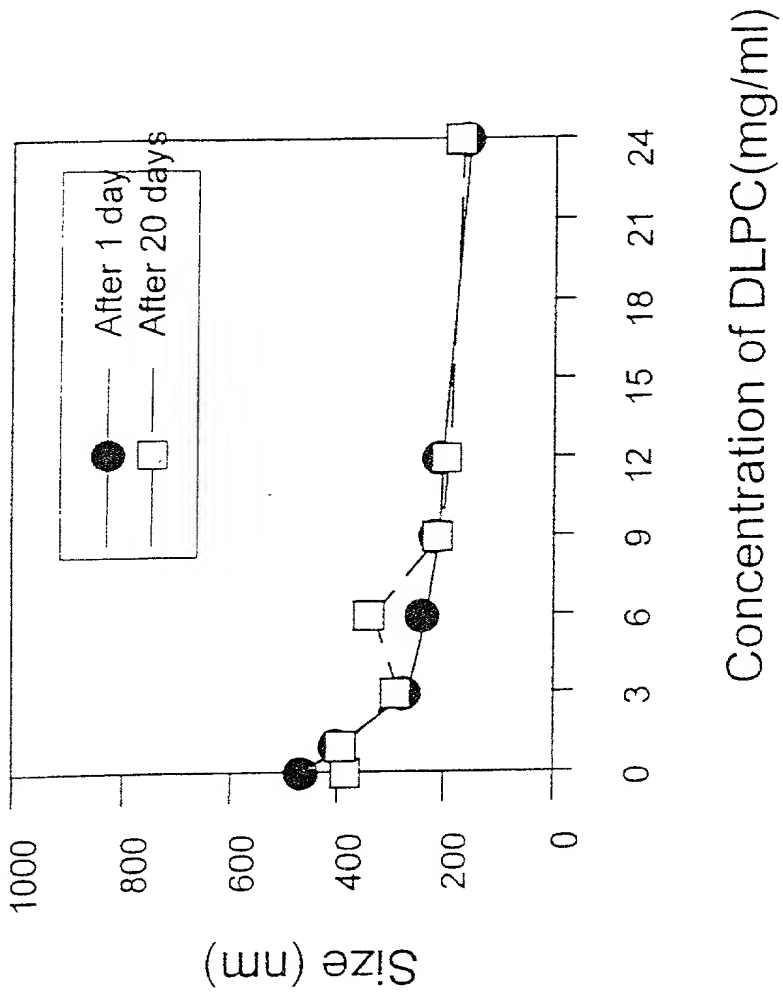

After 1 day
After 20 days
Size (nm)
1000
800
600
400
200
0
0 3 6 9 12 15 18 21 24
Concentration of DLPC(mg/ml)

FIG.3A

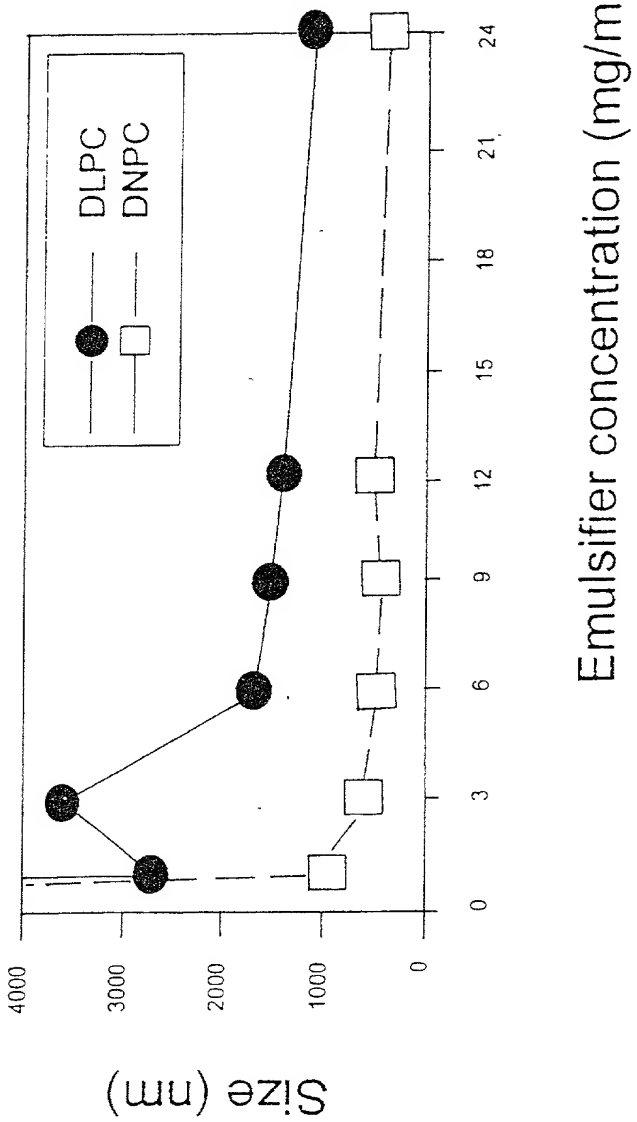

DLPC
DNPC
Size (nm)
4000
3000
2000
1000
0
0
3
6
9
12
15
18
21
24
Emulsifier concentration (mg/ml)

FIG.3B

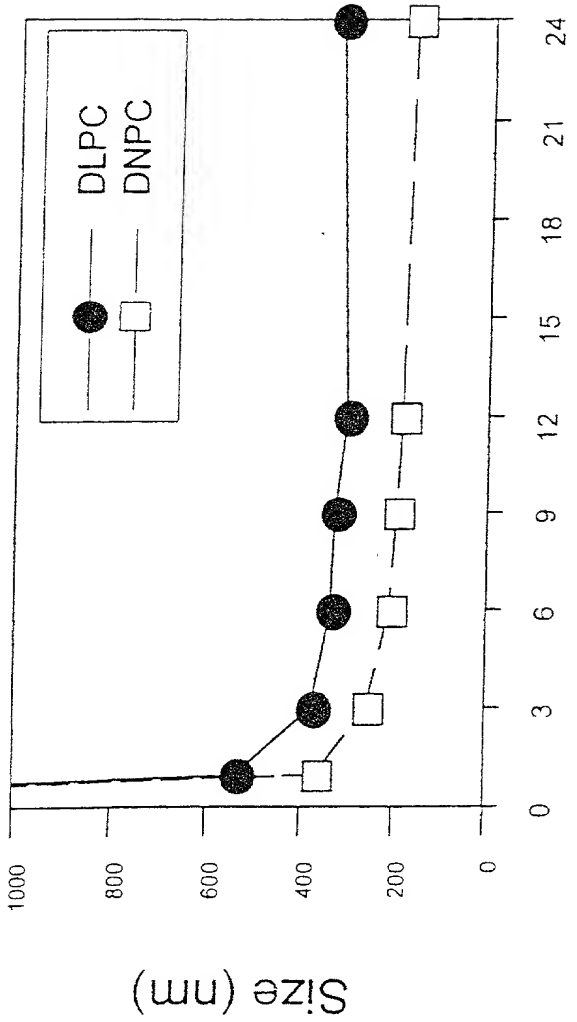

DLPC
DNPC
1000
800
600
400
200
0
0
3
6
9
12
15
18
21
24
Size (nm)
Emulsifier concentration (mg/ml)

FIG.3C

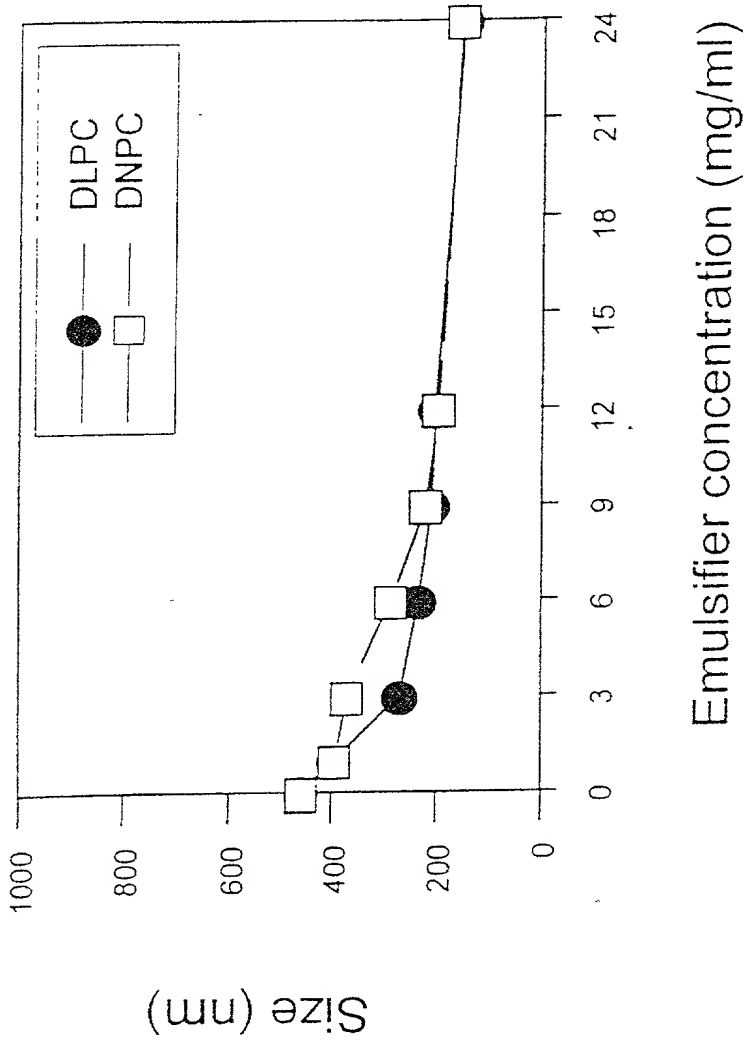

DLPC
DNPC
Size (nm)
1000
800
600
400
200
0
0
3
6
9
12
15
18
21
24
Emulsifier concentration (mg/ml)

FIG.4

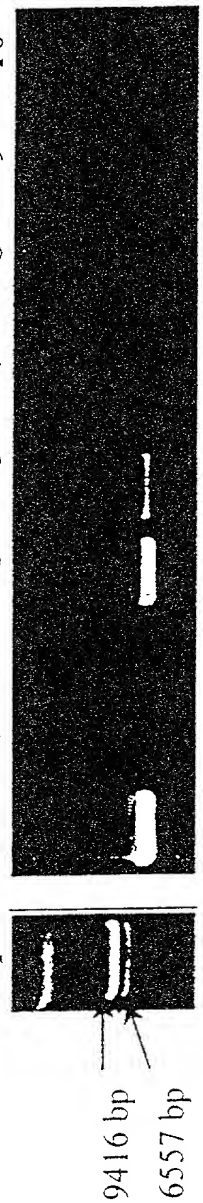


FIG.5

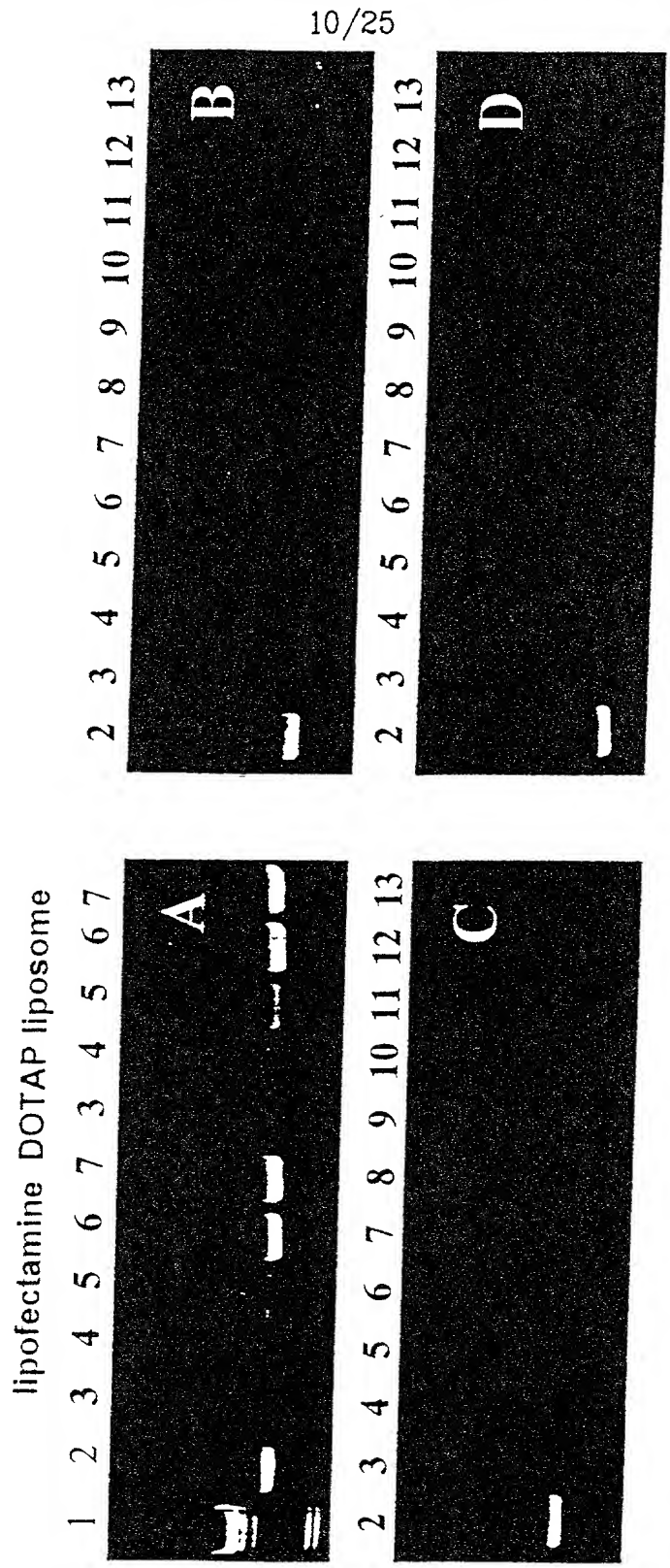

lipofectamine DOTAP liposome
1 2 3 4 5 6 7
A
2 3 4 5 6 7 8 9 10 11 12 13
B
2 3 4 5 6 7 8 9 10 11 12 13
C
2 3 4 5 6 7 8 9 10 11 12 13
D

FIG.6

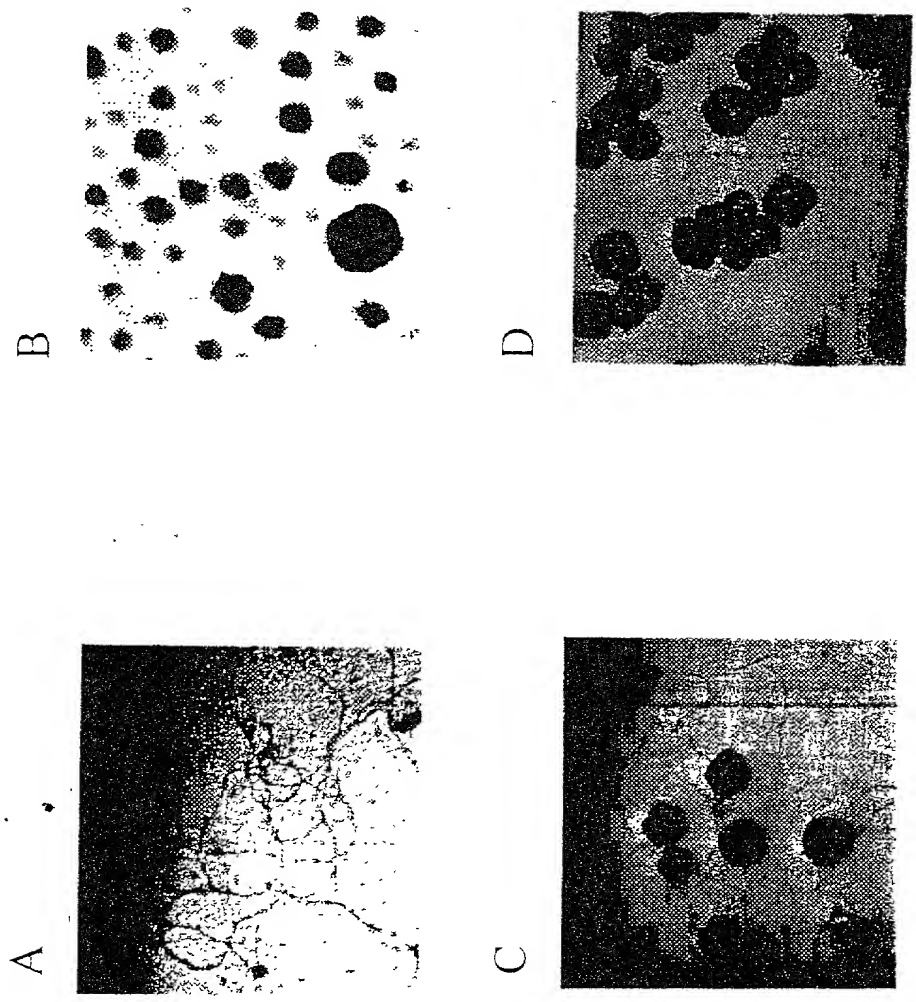


FIG. 7

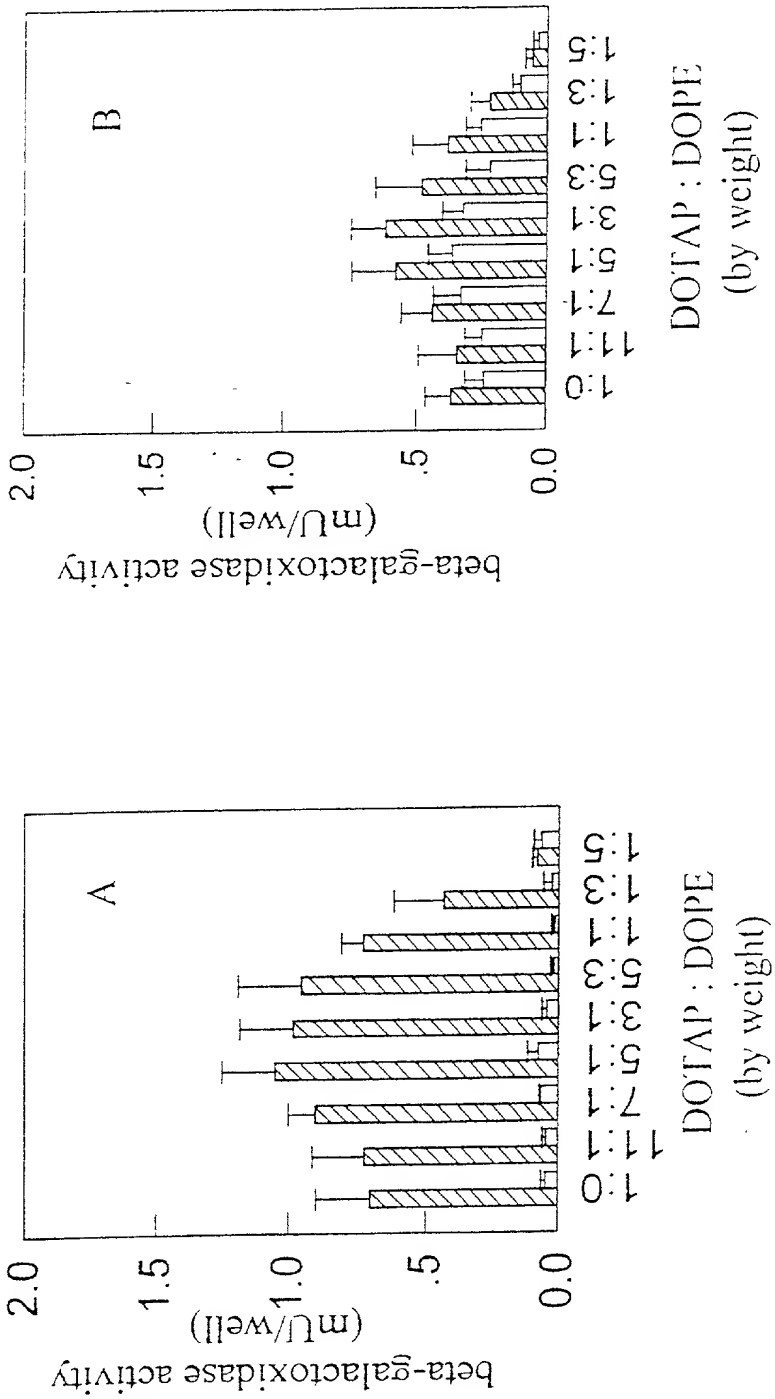

A
B
beta-galactoxidase activity
(mU/well)
2.0
1.5
1.0
.5
0.0
1:0
11:1
7:1
5:1
3:1
5:3
1:1
1:3
1:5
DOTAP : DOPE
(by weight)

FIG.8

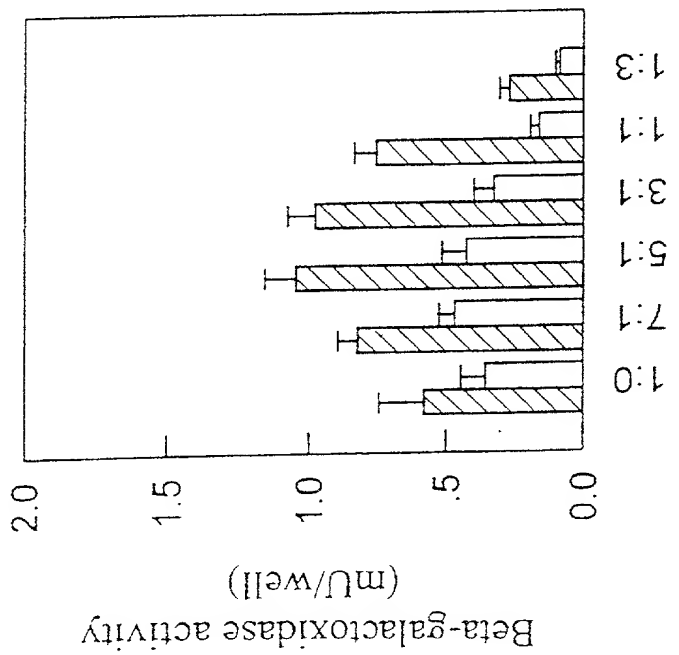

Beta-galactoxidase activity
(mU/well)
2.0
1.5
1.0
.5
0.0
1:0
7:1
5:1
3:1
1:1
1:3
(DOTAP / DOPE 5/1) : DIOLEIN
(by weight)

FIG.9

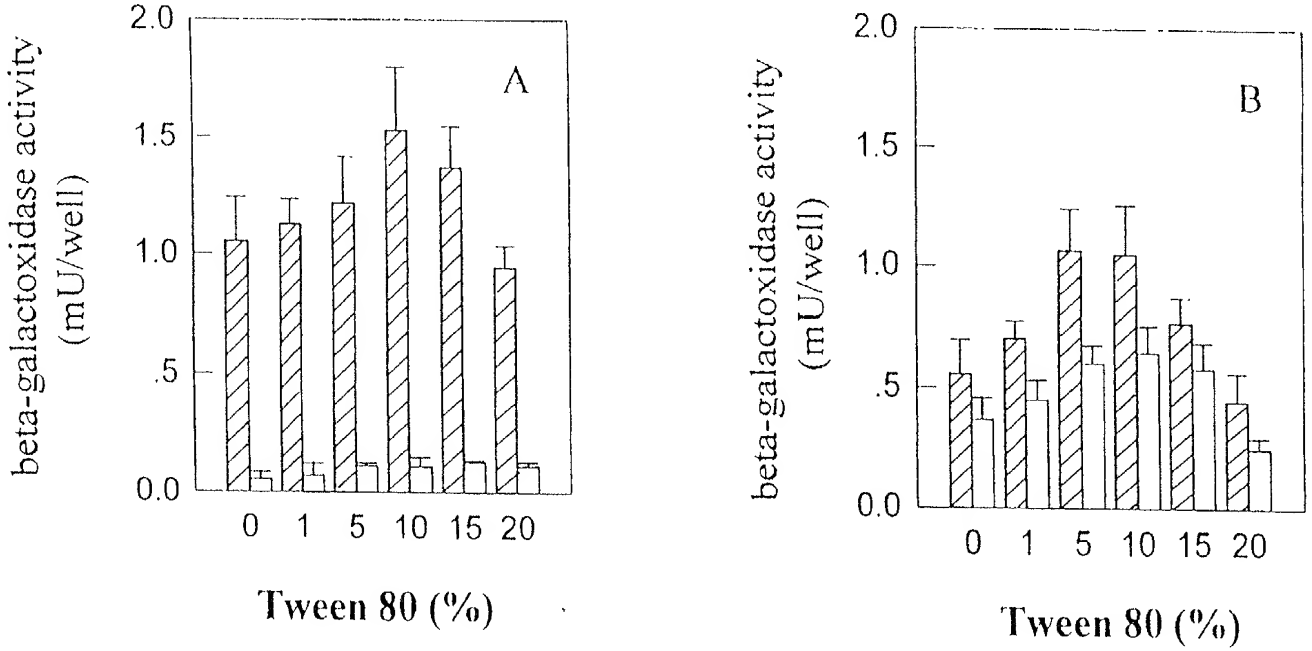

beta-galactoxidase activity
(mU/well)
2.0
1.5
1.0
.5
0.0
A
0 1 5 10 15 20
Tween 80 (%)
beta-galactoxidase activity
(mU/well)
2.0
1.5
1.0
.5
0.0
B
0 1 5 10 15 20
Tween 80 (%)

FIG.10

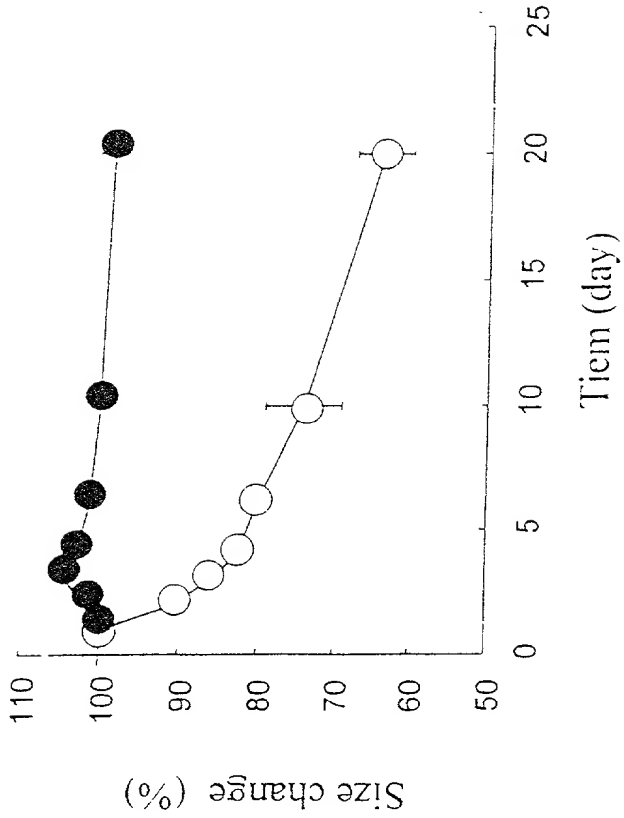


FIG.11

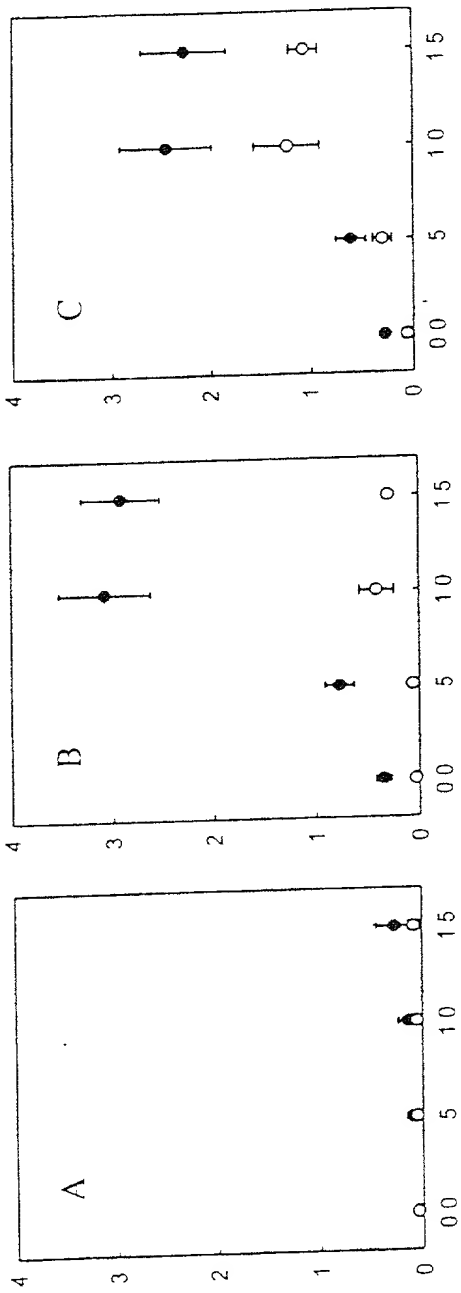

A
B
C
0
1
2
3
4
0
5
10
15
00
beta-galactoxidase activity
(mU/well)
protamine sulfate (μg)

FIG.12

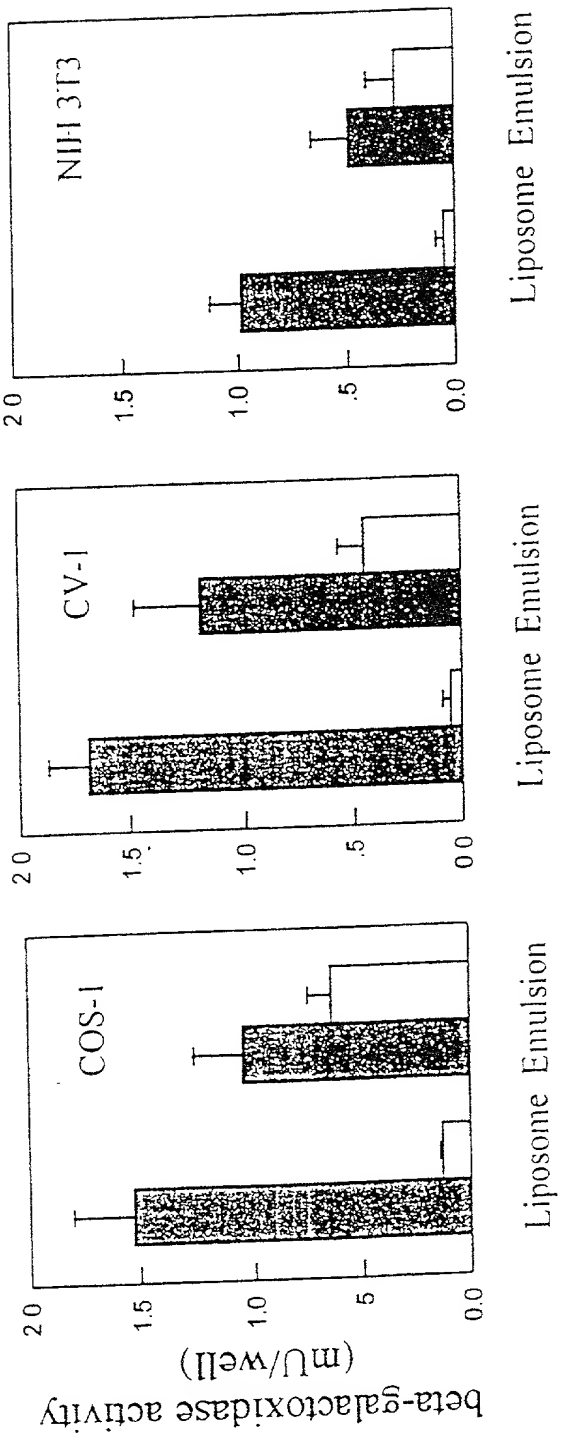

beta-galactoxidase activity
(mU/well)
COS-1
CV-1
NIH 3T3
2.0
1.5
1.0
.5
0.0
Liposome Emulsion
Liposome Emulsion
Liposome Emulsion

FIG.13

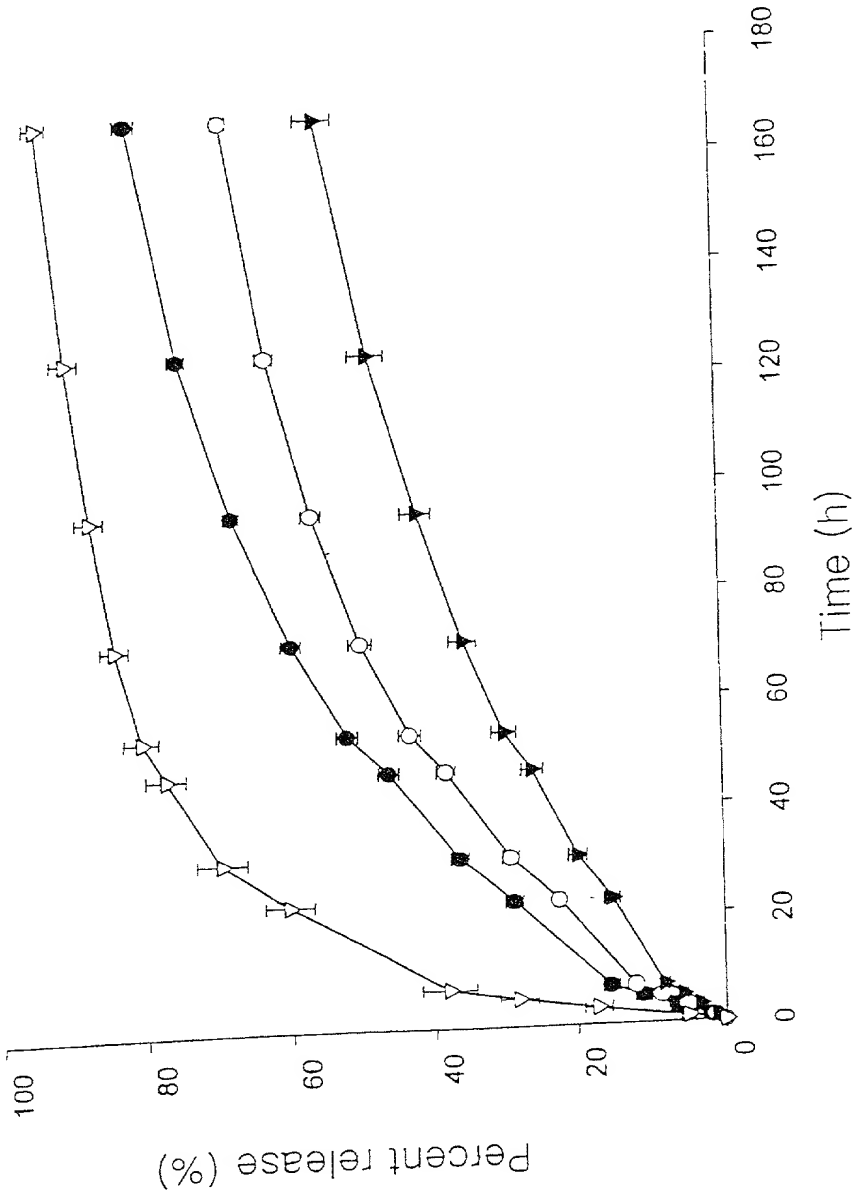
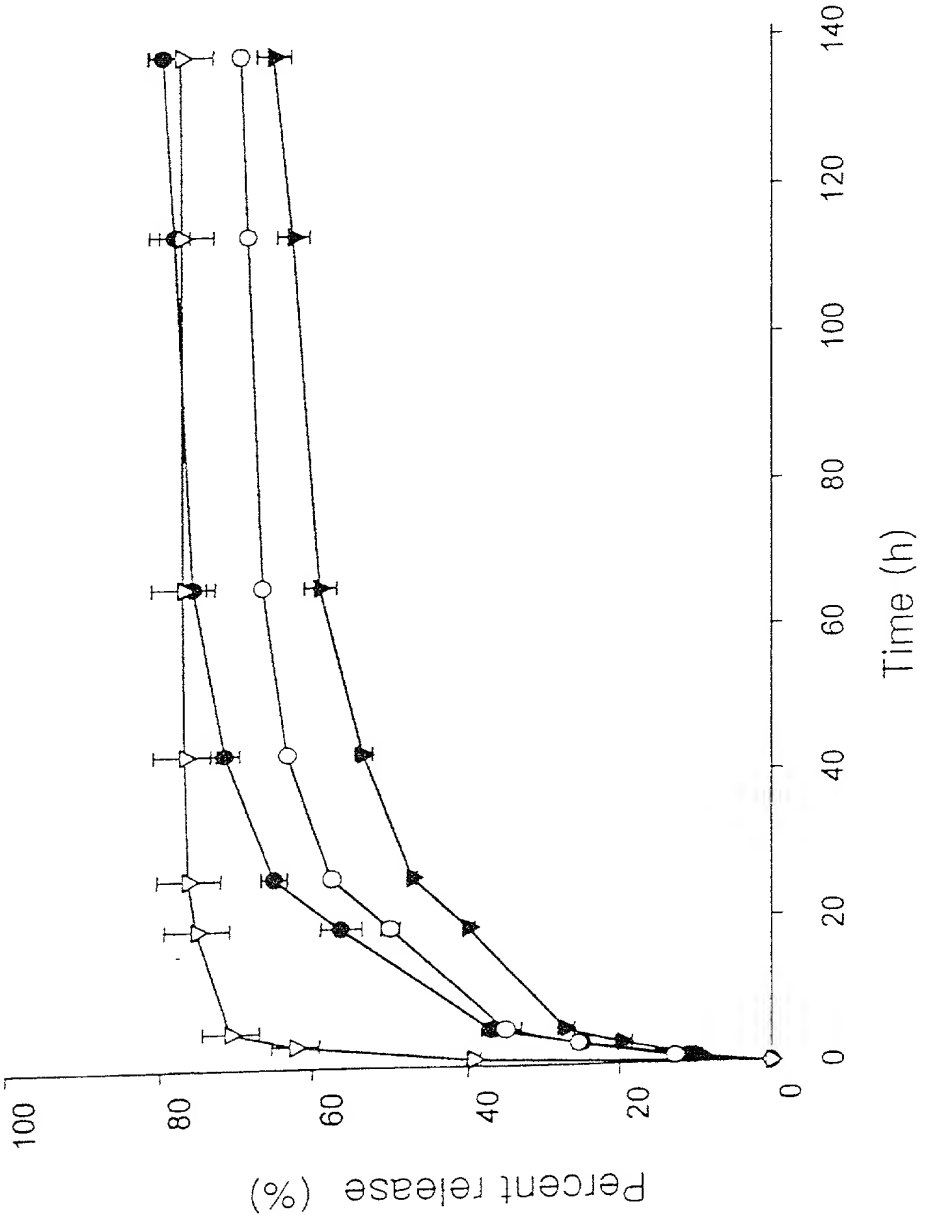

Percent release (%)
0
20
40
60
80
100
Time (h)
0
20
40
60
80
100
120
140
160
180

FIG.14



09/744751
PCT/KR99/00414

FIG.15

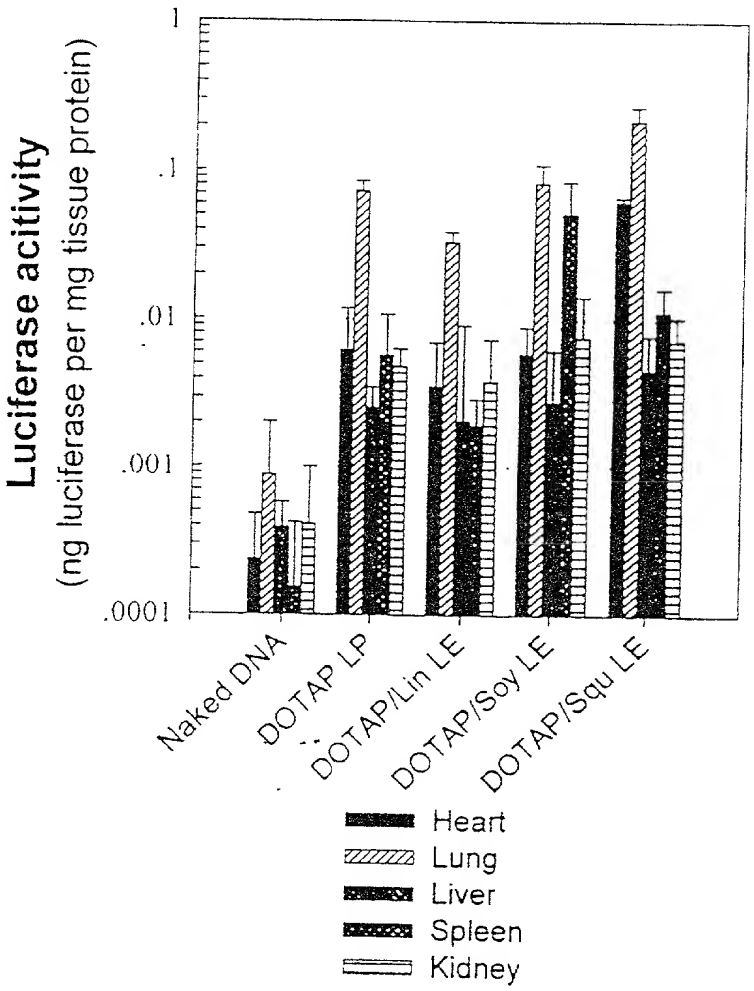


FIG.16

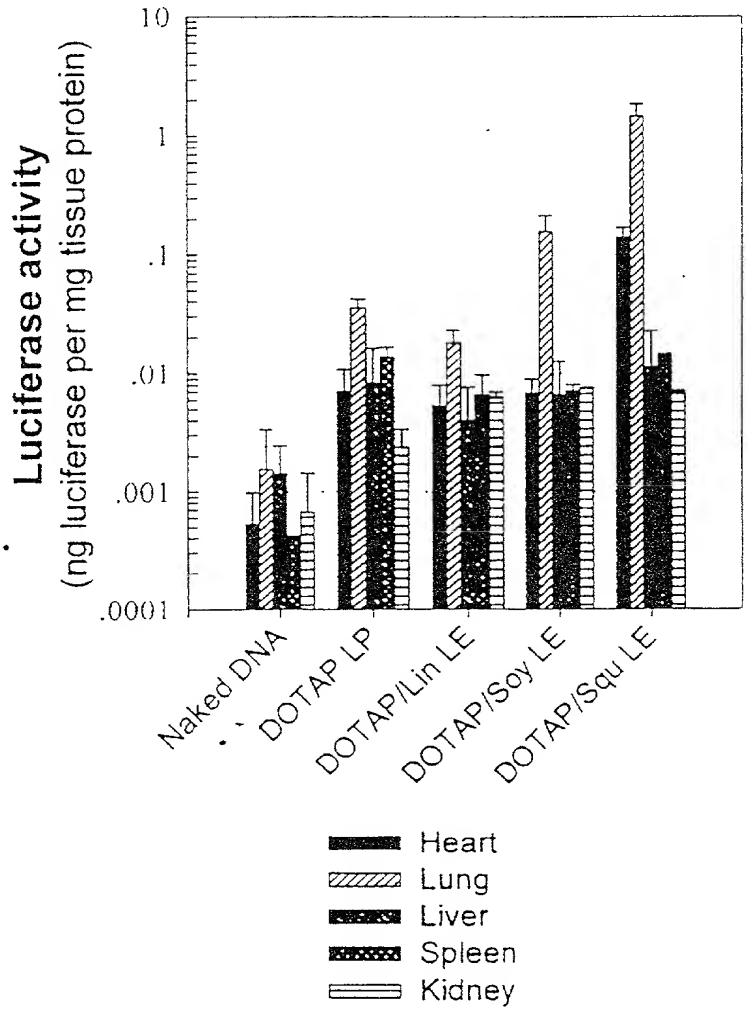

Luciferase activity
(ng luciferase per mg tissue protein)
10
1
.1
.01
.001
.0001
Naked DNA
DOTAP LP
DOTAP/Lin LE
DOTAP/Soy LE
DOTAP/Squ LE
Heart
Lung
Liver
Spleen
Kidney

FIG.17

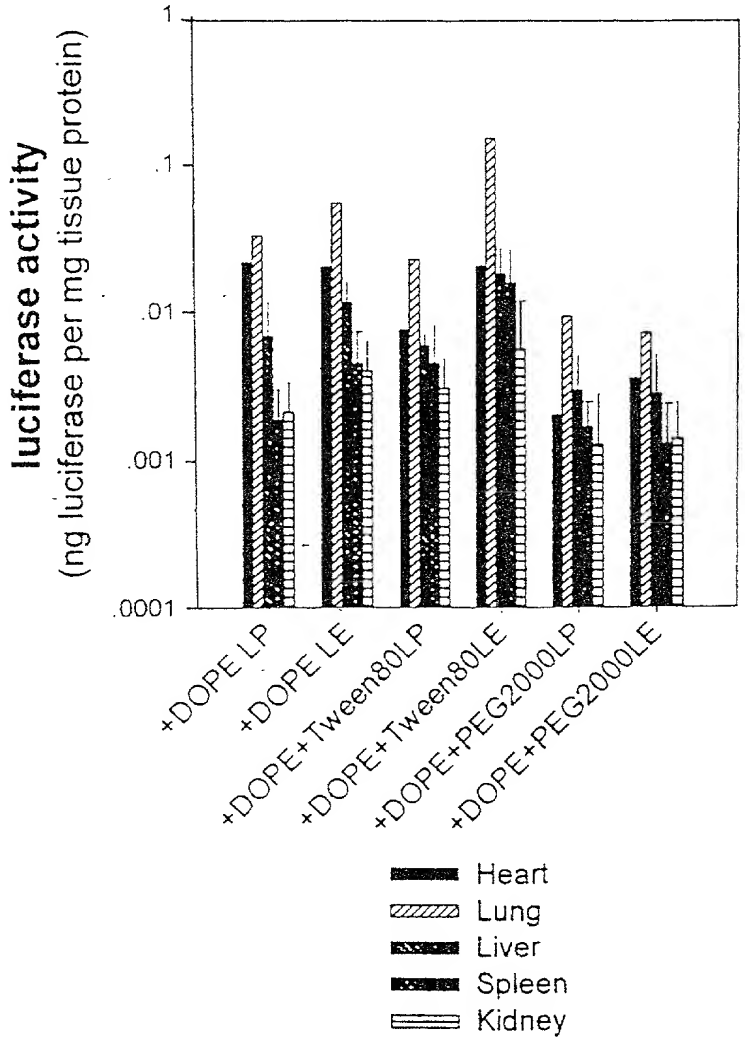

luciferase activity
(ng luciferase per mg tissue protein)
1
.1
.01
.001
.0001
+DOPE LP
+DOPE LE
+DOPE+Tween80LP
+DOPE+Tween80LE
+DOPE+PEG2000LP
+DOPE+PEG2000LE
Heart
Lung
Liver
Spleen
Kidney

FIG.18

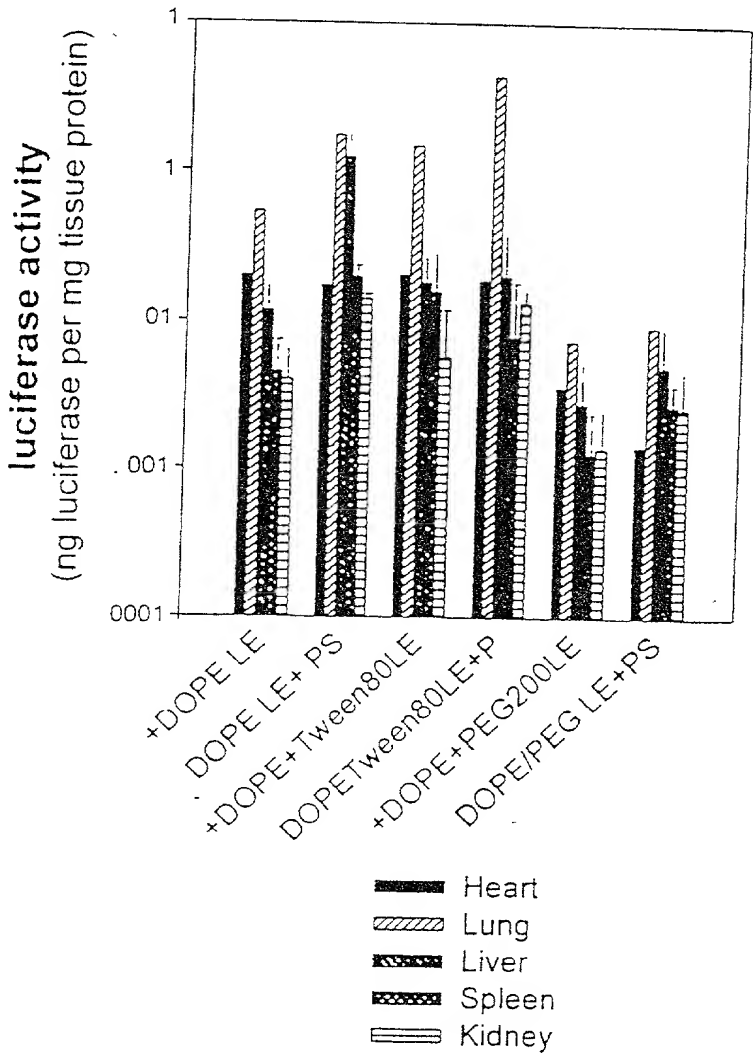

luciferase activity
(ng luciferase per mg tissue protein)
1
1
01
. 001
0001
+DOPE LE
DOPE LE+ PS
+DOPE+Tween80LE
DOPETween80LE+P
+DOPE+PEG200LE
DOPE/PEG LE+PS
Heart
Lung
Liver
Spleen
Kidney

FIG.19

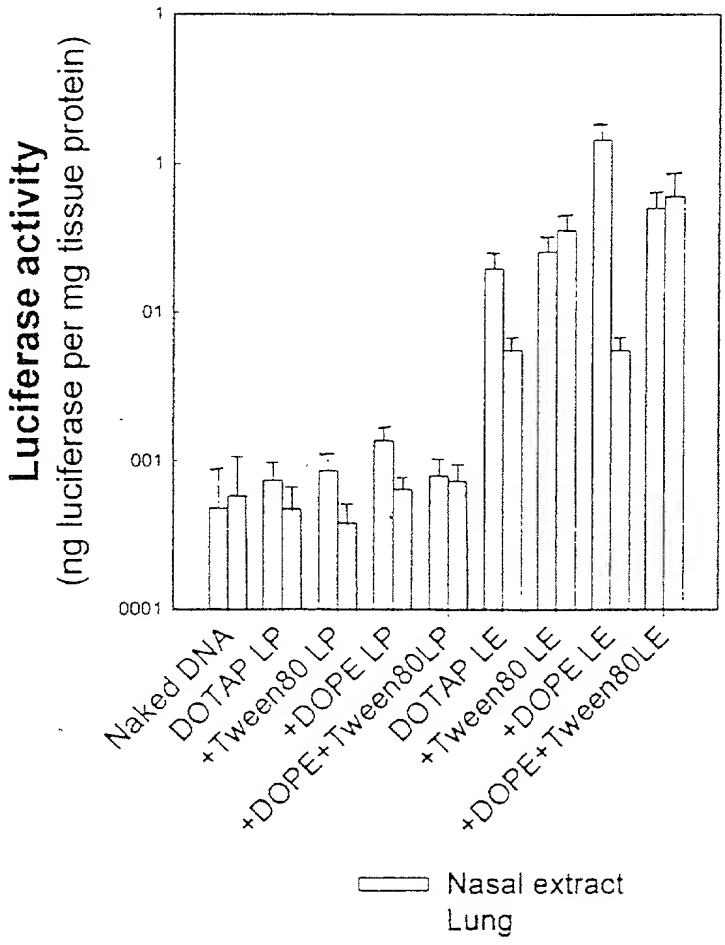
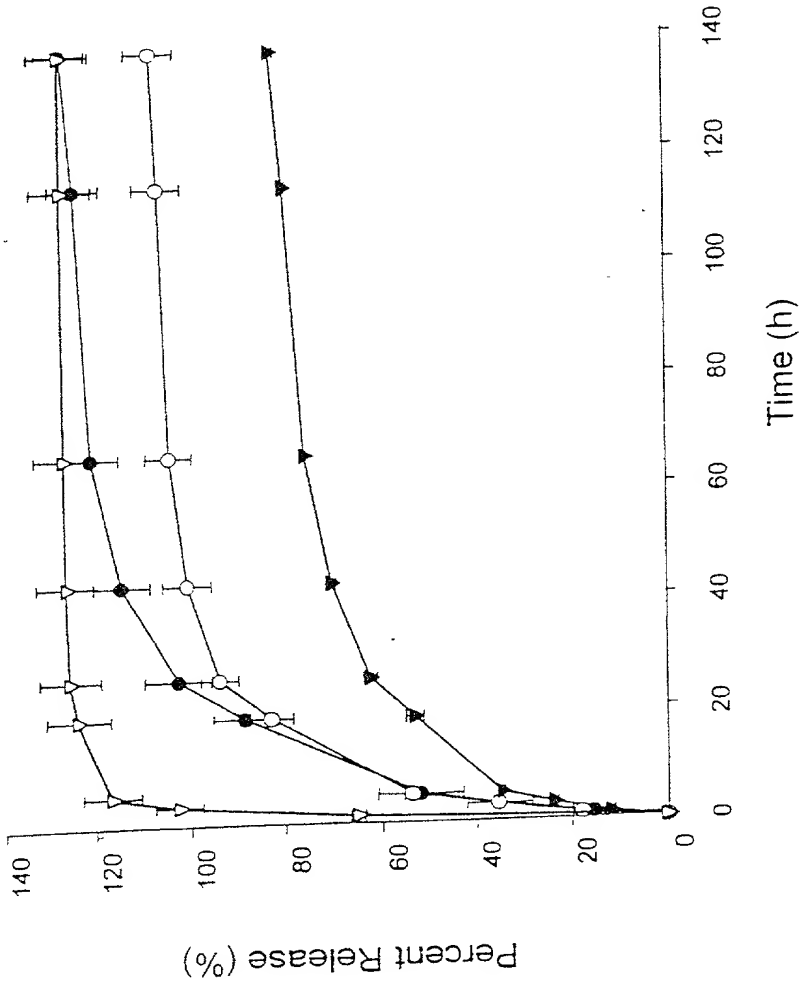

Luciferase activity
(ng luciferase per mg tissue protein)
1
1
01
001
0001
Naked DNA
DOTAP LP
+Tween80 LP
+DOPE LP
+DOPE+Tween80LP
DOTAP LE
+Tween80 LE
+DOPE LE
+DOPE+Tween80LE
Nasal extract
Lung

FIG.20


Percent Release (%)
0
20
40
60
80
100
120
140
Time (h)
0
20
40
60
80
100
120
140

COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below, next to my name.
I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled Lipid Emulsion and Solid Lipid Nanoparticle as a Gene or Drug Carrier the specification of which:

____ is attached hereto.
_X_ was filed on January 29, 2001
as United States Application Number 09/744,751
or PCT International Application Number_____
and was amended on_____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above. I do not know and do not believe that the claimed invention was ever known or used in the United States of America before my invention thereof, or patented or described in any printed publication in any country before my invention thereof or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, and that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months (for a utility patent application) or six months (for a design patent application) prior to this application.

I acknowledge the duty to disclose all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d), of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
1998-31249 (Number)	KR (Country)	31-July-1998 (Day/Month/Year Filed)	X Yes	___ No
PCT/KR99/00414 (Number)	WO (Country)	30-July-1999 (Day/Month/Year Filed)	X Yes	___ No
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	___ Yes	___ No

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below

_____ (Application Number) _____ Filing Date

_____ _____

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Number) Filing Date (Status – patented, pending, abandoned)

(Application Number) Filing Date (Status – patented, pending, abandoned)

(Application Number) Filing Date (Status – patented, pending, abandoned)

I hereby appoint Toni-Junell Herbert, Registration No. 34,348, Mark R. Shanks, Registration No. 33,781, David W. Woodward, Registration No. 35,020, Keith D. Hutchinson, Registration No. 43,687, Joseph G. Contrera, Registration No. 44,628, Chalin A. Smith, Registration No. 41,569, Shelly Guest Cermak, Registration No. 39,571, Suzannah K. Sundby, Registration No. 43,172, of SHANKS & HERBERT, telephone (703) 683-3600, with a mailing address at:

TransPotomac Plaza
1033 N. Fairfax St., Suite 306
Alexandria, VA 22314

with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith.

The undersigned hereby authorizes the U.S. Attorneys named herein to accept and follow instructions from undersigned's assignee, if any, and/or, if the undersigned is not a resident of the United States, the undersigned's domestic attorney, patent attorney or patent agent, as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and the undersigned. In the event of a change in the person(s) from whom instructions may be taken, the U.S. attorneys named herein will be so notified by the undersigned.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

Full Name of Sole/First Inventor: Seo Young JEONG

Inventor's Signature: Seo Young Jeong Date: 01/31/08

Residence: Koyang, Kyungki-do KRX (City, State) Citizenship: Republic of Korea (Country)

Post Office Address: 5, Munchonmaeul Life Apt. 205/501, Juyeop 2-dong, Ilsan-ku, Koyang Kyungki-do 411-371, Republic of Korea

2-0

Full Name of Second/Joint Inventor: Ick Chan KWON

Inventor's Signature: [signature] Date: 01/3/9

Residence: Nowon-ku, Seoul KRX Citizenship: Republic of Korea
(City, State) (Country)

Post Office Address: 274, Shiyoung Apt. 706-704, Hakye-dong, Nowon-ku, Seoul 130-230, Republic of Korea

3-0

Full Name of Third/Joint Inventor: Hesson CHUNG

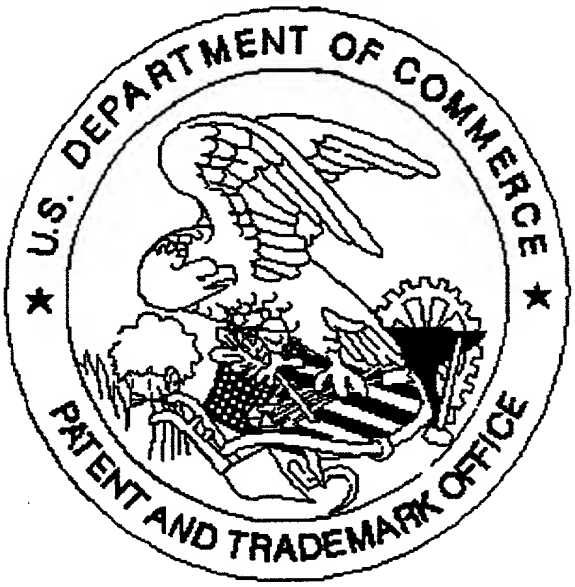
Inventor's Signature: [signature] Date: Mar. 9, 01

Residence: Namdong-ku, Inchon KRX Citizenship: Republic of Korea
(City, State) (Country)

Post Office Address: Jukong Apt. 201-1005, Mansoo-dong, Namdong-ku, Inchon 405-240, Republic of Korea

United States Patent & Trademark Office

Office of Initial Patent Examination -- Scanning Division



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☐ Page(s)_____of_____ were not present for scanning. (Document title)

☐ Page(s)_____of_____were not present for scanning. (Document title)

☑ ***Scanned copy is best available.*** 3 page drawings over dark